Poly(ADP-ribose) glycohydrolase (PARG) is a new therapeutic target for breast cancer

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ABSTRACT

Breast cancer is a heterogeneous disease, but is invariably associated with genome wide deregulation of transcription. The silencing of tumor suppressor genes (TSGs) in particular is thought to be an early, initiating event in many cancers. While re-expressing these genes is a clinically relevant goal, the development of therapeutics targeting the reactivation of TSGs has been hampered by a lack of understanding of the mechanisms responsible for TSG silencing. Recently, however, we have described a novel means through which several TSGs become silenced in cancer, which may highlight new therapeutic targets. This involves the epigenetic regulatory factor Ctcf which is critical for maintaining transcriptional activation and organizing chromatin at several TSG loci. In cells where these TSGs are silenced, the DNA binding of Ctcf to these TSGs is lost. This lack of DNA binding is associated with a loss of the post-translational modification by poly(ADP)ribosylation (known as PARylation). Aberrant dePARylation of Ctcf is associated with breast cancer progression, underscoring the importance of this Ctcf post-translational modification. We have novel data indicating that the dePARylating enzyme Parg is overexpressed in cancer. We hypothesize that inhibition of Ctcf PARylation by Parg disrupts normal epigenetic patterns at TSGs leading to subsequent gene silencing. We propose to examine the impact of Parg overexpression on the epigenetic programming and expression of Ctcf target genes, as well as the proliferation of breast epithelial cells.

To determine the relevance of Parg in breast cancer, we have accumulated evidence from bioinformatics sources and through our own experimentation revealing an enrichment of Parg in a significant proportion of breast cancers. For instance, we have evidence from the Oncomine database and the UCSC Cancer Genome Browser that Parg is overexpressed at the mRNA level in 30-50% of breast cancers. Likewise, at the protein level, higher Parg expression is found in breast cancer cell lines compared to untransformed cell lines and is found to be enriched in more aggressive stages of a mouse tumor model. This is complemented with clinical breast tissue samples showing overexpression of Parg protein in breast cancer.

In support of clinical data, we have generated Parg-overexpressing Mcf10a cells to assess the role of this protein in mediating cellular transformation. Interestingly, Parg overexpression induced cells to shift from an epithelial morphology to a mesenchymal one. This was met with a

decrease in the rate of proliferation *in vitro*. The expression of the Parg transgene, however, was quickly lost and alludes to a significant role for Parg in cellular biology.

Overexpression studies were complemented with drug and knockdown studies. This work illustrated that a shRNA knockdown of Parg was met with a significant decrease in cell growth. Similar results were obtained for cells treated with the Parg inhibitor, tannic acid. Use of this drug caused a decrease in the repressive histone mark H3K27me3 which we believe may restore the expression of silenced TSGs. Likewise, tannic acid induced DNA damage foci over the course of long treatments with the drug, suggesting that DNA damage, too, may contribute to the decrease in cellular proliferation observed. Ultimately, this work provides evidence for a therapeutic benefit of targeting Parg in breast cancer.

RÉSUMÉ

Le cancer du sein est une maladie hétérogène, invariablement associée à une perturbation de l'expression génique. Le silençage des gènes suppresseurs de tumeurs (GST) est l'un des phénomènes jouant un rôle lors de l'initiation du cancer. Si la réactivation de l'expression de ces gènes est une stratégie attractive sur le plan clinique, la mise au point de traitements efficaces a été entravée par la compréhension insuffisante des mécanismes responsables du silençage des GST. Récemment, de nouveaux mécanismes régulant le silençage des GST dans le contexte du cancer ont été décrit, ce qui pourrait conduire à l'identification de nouvelles cibles thérapeutiques. Un des exemples concerne la protéine CTCF, un facteur essentiel au maintien de l'organisation de la chromatine et de l'activité transcriptionnelle à de nombreux loci du génome. Dans des cellules où des GST sont « silencés », la capacité de liaison de CTCF proche de ces gènes est perdue. La perte de cette liaison est due à l'abrogation d'une modification post-traductionnelle sur CTCF : la poly-(ADP)-ribosylation (aussi appelée PARylation). Selon notre hypothèse, l'inhibition de la PARylation de CTCF par l'enzyme PARG, responsable de la déPARylation, perturbe les patrons de modification épigénétiques normaux des GST, ce qui aboutit au silençage de ces gènes. Nous nous proposons d'analyser l'impact de la surexpression de PARG dans le cadre du cancer du sein sur la programmation des modifications épigénétiques et l'expression de gènes cibles de CTCF, ainsi que sur la prolifération des cellules épithéliales mammaires.

Dans un premier temps, nous avons recueilli des données de sources bio-informatiques et de nos propres expériences, et nous avons pu observer une surexpression de l'ARNm de PARG dans 30 à 50% des cas de cancer du sein (UCSC Cancer Genome Browser et Oncomine). De plus, les niveaux protéiques de PARG sont plus importants dans les lignées cellulaires de cancer du sein par rapport aux lignées cellulaires non transformées.

Dans un deuxième temps, nous avons utilisé la lignée MCF10A (immortalisée mais non transformé) pour générer une lignée cellulaire surexprimant PARG afin d'évaluer le rôle de cette protéine dans la transformation cellulaire. Nous avons pu observer que les cellules surexprimant PARG étaient passées d'une morphologie épithéliale à une morphologie mésenchymateuse, et

que leurs vitesses de prolifération avaient diminué. L'expression du transgène PARG a toutefois été rapidement perdue, ce qui laisse entrevoir le rôle significatif de PARG en biologie cellulaire.

Dans un troisième temps, nous avons voulu observer les effets de la perte de PARG sur des lignées de cancer du sein en utilisant soit une drogue (acide tannique : TA) soit la technique des shARNs. Par ces deux méthodes, nous avons montré que la déplétion de PARG (ou inactivation) ralentie significativement la prolifération des cellules cancéreuses. De plus, le traitement avec l'acide tannique conduit d'une part à la formation de nombreux foyers de dommages à l'ADN, et d'autre part à la diminution de la marque répressive H3K27me3, ce qui pourrait conduire à la réexpression de GST. Enfin, ce travail permet de mettre en avant le potentiel de PARG comme cible thérapeutique dans le traitement du cancer du sein.

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1. INTRODUCTION

Breast cancer is an epidemic afflicting approximately one out of every nine women (Kurian et al., 2010). This heterogeneous disease is clinically stratified into four major subtypes; luminal A, luminal B, Her2+ (elevated Her2, Estrogen receptor (ER) negative) and triple-negative (Her2, ER and progesterone receptor (PR) negative). However, current literature indicates further stratification using additional clinical markers, such as cytokeratins, Cyclin D1 and Claudin (Curtis et al., 2012; Gusterson et al., 2005; Prat et al., 2010), will provide more precise prognostic and predictive information. Patients whose tumors fall into the triple-negative category have the poorest clinical outcomes. While these aggressive tumors initially respond to chemotherapy, triple-negative patients are at high risk for metastatic recurrence and have poor overall survival (Di Cosimo and Baselga, 2010). Clearly, new therapeutic strategies are desperately needed to combat triple-negative tumors both at the time of onset and if necessary, at recurrence.

This thesis will focus on detailing a new mechanism for breast cancer treatment that may provide relief to some women affected by the disease. Specifically, it will focus on a new mechanism of re-expressing epigenetically silenced tumor suppressor genes in order to restore growth control in malignant cells.

1.1 | Strategies for breast cancer therapy

Research in the field of breast cancer has provided several new therapeutic alternatives since the advent of chemo- and radiation treatment. These have come in the form of targeted therapy directed towards ER and Her2 and have been met with much success in the clinic. Drugs like tamoxifen (directed against ER) and trastuzumab (directed against Her2), combined with chemotherapy and radiation have resulted in huge improvements in breast cancer survival (Group 2001, Slamon, Leyland-Jones et al. 2001, Jordan 2002). This success highlights the promise of targeted therapy for the clinic.

Targeted therapy is concerned with regulating the expression or function of two broad categories of factors termed oncogenes and tumor suppressor genes (TSGs) (Sawyers 2004, Pegram, Pietras et al. 2005). Understanding the distinct role of these factors in promoting

carcinogenesis is important to define new therapeutic targets. As such, we will proceed with an in-depth look at the factors involved in oncogenesis before attempting to discuss their potential for therapy.

1.2 | Oncogenes

One critical class of factors involved in carcinogenesis is 'oncogenes'. Oncogenes promote cancer initiation and progression following gain-of-function mutations or increased expression levels in the cell (Land, Parada et al. 1983). There are several different mechanisms through which oncogenes can act to induce malignancy. For instance, oncogenes can work by inducing cells to undergo proliferation. This is one of the ways through which the Her2 protein contributes to breast cancer (Osborne, Wilson et al. 2004). Specifically, Her2 acts in a ligand-independent manner through the MAPK and PI3K pathways to promote the transition from the G1 to S phase of the cell cycle (Zaczek, Brandt et al. 2005). By maintaining downstream signaling effectors in an active state, upregulation of Her2 reduces the need for growth factors and in turn, promotes cancer progression. Alternatively, some oncogenes are involved in promoting cell survival through the inhibition of apoptosis (Kerr, Winterford et al. 1994). An example of this is the classic B-cell lymphoma 2 protein (Bcl-2). This factor can prevent the release of one of the key initiators of apoptosis, cytochrome c, from the mitochondria (Yang, Liu et al. 1997). Cytochrome c is an important factor in caspase activation, a key factor driving the process of controlled cell death. In this way, Bcl-2 favors cell survival and allows cells to proliferate uncontrollably. Ultimately, this contributes to the malignancy of the disease. Additionally, oncogenes can play a role in metastasis by positively affecting the invasive and migratory properties of cells or by driving angiogensis (Yokota 2000). Ras, for instance, has been implicated in both processes, described as a factor promoting metastasis through its effect on the cytoskeleton-organizing protein Rac (Campbell and Der 2004) and as a factor promoting the expression of VEGF which favors blood vessel growth (Rak, Mitsuhashi et al. 1995). While there are clearly various mechanisms through which oncogenes can act, the ultimate outcome of these is to provide an increased growth and survival advantage to cells.

1.3 | Tumor suppressor genes

In addition to the overexpression or increased activity of oncogenes, cancer can also result from the deletion, loss-of-function or silencing of TSGs. TSGs are protective factors that restrict the oncogenic process (Osborne, Wilson et al. 2004). Mutations or silencing of such genes contributes to cancer initiation and progression. These alterations allow cells to grow uninhibited, despite any abnormalities and defects that they may have acquired (Weinberg 1991). Specifically, these factors can be involved in restricting cell cycle progression ($p15^{INK4b}$, $p16^{INK4a}$), promoting DNA damage repair (Brca1, Brca2), inducing apoptosis (p53), promoting cell adherence (CDH1) and assisting in detoxification (GSTP1) (Esteller 2002). For instance, the TSG $p16^{INK4a}$ is involved in regulating cell cycle progression. By binding cyclin-dependent kinases 4/6, p16 prevents the phosphorylation of targets such as the retinoblastoma protein (Rb). In this way, Rb can inhibit the transcription factor E2F from activating genes responsible for cell cycle progression (Liggett Jr and Sidransky 1998). Other TSGs like Brca1 and 2, on the other hand, are responsible for homologous recombination reactions in response to double-strand DNA damage. These factors ultimately prevent erroneous repair of the DNA to prevent the integration of potentially oncogenic lesions in the genome (Venkitaraman 2001). The loss of growth control is critical for the manifestation of cancer, and is therefore an intriguing target for management of the disease.

1.4 | Cancer progression model

The progression of cancer is believed to be multifactorial, resulting from the accumulation of multiple lesions over time involving both oncogenes and TSGs. This is evidenced by the fact that older individuals have a higher probability of developing cancer, given the longer time they have had to acquire genomic insults (Miller 1980). Initially, a cell begins with one insult, with no significant effects on growth. The second hit contributes to benign tumor formation. The third is thought to confer an increased proliferative capacity to the cell. In the end, the acquisition of multiple hits in the cell promotes a metastatic cell phenotype, leading to malignancy (Vogelstein and Kinzler 1993). The sum of all the activating mutations of oncogenes and the loss-of-function mutations of TSGs ultimately governs whether a cell will

progress towards cancer. This is illustrated in cell culture models. For instance, the human mammary epithelial cells (HMECs) differ from the variant HMECs (vHMECs) in that the latter has silenced $p16^{INK4a}$ and *HOXA9* genes. The progression of these cells towards malignancy is the result of promoter methylation at an increased number of TSG loci including *RASSF1A and SFRP1.* Overexpression of *RAS* in these cell lines promotes senescence of HMECs but increased proliferation of vHMECs. Over time, the *RAS*-overexpressing vHMECS present with more significant genomic instability than the vHMECs. Still, this is not sufficient for these cells to induce tumors *in vivo,* suggesting that still other mutations are required to achieve full malignancy (Dumont, Crawford et al. 2009). Overall, this model illustrates the importance of acquiring multiple lesions for disease progression, with any one particular hit, alone, being insufficient to cause disease. This pattern is replicated *in vivo*, having been particularly well described in colon cancer. In this case, pre-malignant disease is characterized by silencing of such TSGs as *APC* and *p53*. This is followed by the activation of multiple oncogenes including *KRAS* and *EGFR* (Fearon 2011). Thus, as illustrated *in vitro* and *in vivo,* it is clear that multiple hits are required for full-blown carcinogenesis to ensue. This opens the door to a vast consortium of therapeutic targets to be explored.

1.5 | The potential of TSG re-expression for cancer therapy

For the purpose of this project, we have decided to investigate the possibility of TSG restoration as a mechanism for breast cancer therapy. In the past, the loss of TSGs expression in cancer was described to result from genetic defects including gene deletion, mutation and recombination (Levine 1993). Such changes are considered permanent as they are not easily reversible by currently available technologies. However, seminal work by Steve Baylin's group revealed that oftentimes, TSGs are silenced and not deleted or functionally compromised (Merlo, Herman et al. 1995). These changes are thought to occur from epigenetic deregulation in the cell and are considered reversible. Thus, developing ways of re-expressing silenced TSGs is an intriguing and feasible new approach for therapy. The use of epigenetic drugs for therapy is an important new field of research in oncology and is described in further detail in a later section. First, however, we will discuss transcriptional regulation in cells, including the impact of epigenetics, and how this can go awry in cancer.

1.6 | Transcriptional regulation and epigenetics

Gene expression is regulated by controlling access of the RNA polymerase II (RNA pol II) to gene promoters (Li, Carey et al. 2007). The restriction is imposed by the fact that DNA is closely associated to structural proteins termed histones, rather than being free-floating in cells. Specifically, ~146 base pairs of DNA are wrapped around a histone octamer core composed of 2 copies of the histones H2A, H2B, H3 and H4 (Fischle, Wang et al. 2003). This structural entity is referred to as a nucleosome. The density of nucleosomes over a stretch of DNA is an important factor regulating gene expression. Stretches of DNA with low nucleosome density enable gene expression and are regions referred to as euchromatin, while regions of DNA with high nucleosome density are transcriptionally hindered and are referred to as heterochromatin (Lennartsson and Ekwall 2009). The establishment and maintenance of these domains is referred to as epigenetic regulation.

Epigenetic changes are known as mitotically stable modifications to chromatin that modulate gene expression (Goldberg, Allis et al. 2007). These changes do not affect the coding of the DNA, but rather, they act as regulators of transcription. Nucleosomes act as physical barriers for the necessary transcription factors to bind gene promoter regions (Li, Carey et al. 2007). Likewise, DNA methylation restricts the access of important factors required for gene expression. Gene activity can be regulated by modifying methylation of the DNA template or by altering nucleosome positioning. These changes ultimately impact the accessibility of the DNA to RNA pol II. Together, these changes are the basis of epigenetic regulation in the cell.

1.7 | DNA Methylation

DNA methylation is among the first epigenetic modifications described. This modification is the result of the addition of a methyl group to cytosine residues that are followed by guanine nucleotides (CpG sequences) (Moore, Le et al. 2012). The enzymes that regulate the addition of this modification are the DNA methyltransferase enzymes (Dnmts) and include the maintenance methylase Dnmt1 and the *de novo* methylases Dnmt3a and 3b. Using S-adenosylmethionine (SAM) as a substrate, Dnmt1 duplicates methylation patterns from parent to daughter DNA strands (Detich, Ramchandani et al. 2001) while the *de novo* methylases are more

promiscuous in their action, adding methylation marks to random CpG sites on the DNA (Okano, Bell et al. 1999). Initially, it was thought that the main role of DNA methylation was to provide a direct physical barrier limiting the access of transcription factors to the DNA template (Kass, Pruss et al. 1997). This was the mechanism proposed to explain the effect of DNA methylation on gene silencing and was supported by findings of impaired binding of some transcription factors such as NF-kB and c-Myc to methylated DNA sites (Eden and Cedar 1994). A later study, however, showed that differentially methylated template strands can both be transcribed shortly after microinjection into *Xenopus* oocytes. It is only after a time delay that the methylated strand becomes more closely associated to nucleosomes and becomes transcriptionally silent (Kass, Landsberger et al. 1997). This suggests that DNA methylation is mainly involved in recruiting transcriptional repressors to sites on the DNA, essentially hindering RNA pol II mediated transcription of genes. Indeed, it has been shown that the methyl-CpG binding domain protein MeCP2 recruits the repressive histone deacetylase (described in further detail below) to methylated promoters which, in turn, is believed to promote gene silencing (Jones, Veenstra et al. 1998).

A paradox exists when considering the fact that CpG sites are ideal targets for methylation, but these CpG elements are abundant at gene promoters. Specifically, it is estimated that between 60 to 90% of CpG pairs are methylated in the adult genome (Siegfried and Cedar 1997). Moreover, there is an enrichment of CpG islands in promoter regions of 70% of genes (Saxonov, Berg et al. 2006). Interestingly, there exist stretches of DNA with a significant enrichment of repeating C and G nucleotide pairs, particularly at transcription start sites (Carninci, Sandelin et al. 2006). These regions are termed CpG islands and can extend from 500 to thousands of base pairs in length. Although CpG residues are ideal targets for methylation, CpG islands are found hypomethylated under normal cellular conditions (Antequera 2003). The reason for this remains unclear to this date. Regardless of the mechanism by which CpG islands are spared, the hypomethyaltion at these sites is important to maintain a transcriptionally active state in the cell, as these CpG islands often reside. The localization of this methylation-sensitive sequence at promoters may be important for the timely control of genes through regulation of methylation levels during development. The existence of these sites implies that important regulatory mechanisms are likely in place to keep CpG islands unmethylated. Deregulation in

this process can lead to problems in such processes as cancer, which will be further discussed below.

1.8 | Chromatin-modifying proteins and remodelers

In addition to promoter methylation status, the nucleosome positioning at these sites is another important factor in transcriptional regulation (Li, Carey et al. 2007). Among the proteins factors involved in regulating nucleosome distribution are the chromatin-remodeling and chromatin-modifying proteins. Chromatin remodelers are involved in regulating the positioning of nucleosomes on the DNA template (Owen-Hughes 2003). This process is energy dependent, requiring the hydrolysis of ATP by the enzyme to disrupt the association between the DNA and histones in order to remove or displace nucleosomes (Clapier and Cairns 2009). Nucleosome restructuring can be achieved by such proteins as the SWI/SNF family of remodelers. In drosophila, the SWI/SNF analog Brahma is associated with euchromatin throughout the genome and is important for RNA pol II-mediated transcription (Armstrong, Papoulas et al. 2002). This illustrates how chromatin remodelers can be intimately linked with gene expression in the cell.

The specific localization of chromatin-remodeling enzymes is affected by the action of chromatin-modifying enzymes. These proteins add post-translational modifications to histone tails, extensions of the proteins that protrude from the nucleosome. The accumulation of post-translational modifications on histone tails is referred to as the "histone code" (Jenuwein and Allis 2001). These changes affect the docking of various proteins (including chromatin remodelers) to the DNA (Clapier and Cairns 2009). The contribution of all the factors recruited to the histones leads to either active recruitment of RNA pol II to the transcription start site of genes, or lack thereof (Li, Carey et al. 2007). Here below, we describe the role of histone modifications and their impact on transcription.

1.8.1 | Histone acetylation

Acetylation of histones involves the catalyzed addition of an acetyl group from an acetyl-coenzyme A substrate onto lysine residues by histone acetyltransferases (HATs). The removal of this mark, on the other hand, involves the antagonistic histone deacetylase enzymes (HDACs).

Part of the role of acetylation is the neutralization of the positive charge of the lysine amino acid in the histone tail. This disrupts the electrostatic interactions between the positively charged lysines in histones and the negatively charged DNA, promoting a loosened chromatin structure (Shahbazian and Grunstein 2007). This alludes to a direct role for acetylation in driving gene expression and is consistent with findings that acetylation of lysine 122 on histone 3 (H3K122ac) alone can promote transcriptional activation (Tropberger, Pott et al. 2013). More importantly, however, is the role of histone acetylation in providing a docking site for transcription activating genes (Fischle, Wang et al. 2003). Several transcription factors as well as chromatin remodelers possess a bromodomain, a functional motif that is involved in the recognition of acetylated lysines (Zeng and Zhou 2002). This domain has been shown to be important for such proteins as the chromatin remodeler SWI/SNF to bind acetylated lysines on H3 and H4 and has proven to be vital for proper protein function *in vivo* through the maintenance of transcriptionally active chromatin throughout the genome (Hassan, Prochasson et al. 2002). Not surprisingly, histone acetylation marks are typically found at active promoter sites (Brown, Kennedy et al. 2008). Overall, it is apparent that whether through the disruption of electrostatic interactions between the DNA and histones or through the recruitment of other factors, lysine acetylation plays a key role in gene activation.

1.8.2 | Histone Methylation

Unlike histone acetylation, histone methylation can have different effects on transcription depending on the residue modified. Histone methylation can take on several forms including mono-, di- and tri-methylated states and has been detected on both lysine and arginine residues (Kouzarides 2002). Thus, the outcome of methylation depends on the specific residues affected and the extent of the modification. The same modification on a different residue can lead to a vastly different effect on transcription. For instance, methylation at sites H3K9, H3K27 and H4K20 favor transcriptional repression while methylation at H3K4, H3K36 and H3K79 favor transcriptional activation (Kouzarides 2002, Martin and Zhang 2005). The enzymes involved in this process are the histone methyltransferases (HMTs) which catalyze the addition of the methyl marks and the histone demethylases which are responsible for the reverse reaction (Kouzarides 2002). Generally, it is believed that unlike histone acetylation that can have a direct effect on

transcription, histone methylation is solely involved in mediating recruitment of other proteins for this purpose. The role of the factors that bind the histone modifications is what ultimately dictates the effect on gene expression.

The heterogeneity of the outcome of histone methylation can be exemplified by considering some of the specific examples encountered in the literature. For instance, the epigenetic silencing mark H3K27me3, generated by the EZH2 protein, a member polycomb repressive complex 2 (PRC2) (Morey and Helin 2010), has been linked to various processes in the cell including X-chromosome inactivation, imprinting and gene silencing during differentiation, although the specific mechanisms by which this is achieved are still unclear. The repressive H3K9me modification, on the other hand, is generated by the Suv39H1 HMT protein in humans and has a more straightforward role. This modification serves as a docking site for the heterochromatin protein 1 (HP1), which, in turn, mediates the recruitment of Dnmts as a mechanism of transcriptional repression (Smith and Shilatifard 2010). Alternatively, H3K4me is an important histone activating mark generated by the Mixed-Lineage Leukemia (MLL) protein in humans and can occur in a mono-, di-, or tri-methylated form. Throughout the genome, a high density of H3K4me2 and H3K4me3 can be detected in transcriptionally active promoter regions and H3K4me1 can be detected at enhancer sites (Barski, Cuddapah et al. 2007, Heintzman, Stuart et al. 2007). The transcription-promoting modification H3K36me3, catalyzed by the Set2 HMT, on the other hand, can be found downstream of transcription start sites, even nearing the 3' end of genes (Shilatifard 2006). The H3K36me3 mark has been linked to histone deacetylation to facilitate the processivity of RNA pol II during transcription. Evidently, histone methylation can have a large variety of effects depending on the context in which they are found.

1.8.3 | Cooperation of epigenetic marks

There appears to be an intimate link between epigenetic marks within the cell. For instance, it has been found that the H3K4me3 mark acts as a binding site for the NuA3 HAT complex, stimulating the addition of the activating H3K14 acetylation mark (Taverna, Ilin et al. 2006). Likewise, we see cooperation of DNA methylation and histone modifications in the promoter region of the $p14^{ARF}$ and $p16^{INK4a}$ genes. Specifically, the proteins MeCP2 (Nguyen, Gonzales et al. 2001) and MBD2 (Magdinier and Wolffe 2001) bind upstream methylated CpG

islands and recruit HDACs (Nan, Ng et al. 1998, Dhasarathy and Wade 2008) which act to promote gene silencing at this locus. Methylation of the CpG island within the *BRCA1* gene has also been described to be linked to histone hypoacetylation (Rice and Futscher 2000). This is likely related to findings of direct associations between Dnmts and HDACs (Fuks, Burgers et al. 2000, Robertson, Ait-Si-Ali et al. 2000).

Overall, there is a clear picture that DNA methylation and post-translational histone modifications act in concert to generate one of two outcomes at a given gene promoter: transcriptional activation or silencing. These regulatory mechanisms, however, can go awry in cancer, leading to erratic genetic profiles with a preference for cell survival.

1.9 | Epigenetics and oncogenesis

The first association between epigenetics and cancer was the discovery of altered DNA methylation patterns in malignant cells (Lapeyre and Becker 1979). Early findings revealed global hypomethylation of the genome in tumors, which was thought to promote the reactivation of oncogenes that were silenced under normal conditions (Feinberg and Vogelstein 1983). Genes such as *H-RAS* and *MYC* were discovered to be hypomethylated in cancer which supported this hypothesis (Feinberg and Vogelstein 1983, Sharrard, Royds et al. 1992). Despite this, however, these oncogenes were not found to be overexpressed in the corresponding cancer cells. Thus, the model proposed failed to explain the phenotype observed and this concept was eventually modified. It is now known that cancer cells are marked by a global shift in the epigenome from a hypermethylated state to a hypomethylated one, with the opposite effect occurring at gene promoters (Gama-Sosa, Slagel et al. 1983). Specifically, there is a trend towards increased methylation of CpG islands, found within gene promoter regions in cancer (Gardiner-Garden and Frommer 1987). The mechanism for this shift is unclear, although the implications of these changes are well understood. The hypomethylation of genes exposes DNA coding regions to allow for increased susceptibility to UV and other mutagens, exposes repetitive sequences that promote genetic recombination events and exposes alternative reading frames for the generation of inappropriate gene transcripts (Gaudet, Hodgson et al. 2003, Daskalos, Nikolaidis et al. 2009). These malignant changes are met with CpG promoter methylation, causing the silencing of

hundreds of genes, including TSGs (Jones and Baylin 2007). Thus, epigenetic deregulation in cancer favors pro-tumorigenic changes while downplaying cell cycle control checkpoints and apoptotic factors, leading to disease progression.

The landmark discovery of TSG promoter methylation took place in the late 1980s where aberrant CpG methylation of the retinoblastoma gene (*RB*) promoter was described (Greger, Passarge et al. 1989). At that time, however, the implications of this change were still not well understood. It was only a few years later that the notion of promoter methylation promoting TSG silencing and oncogenesis was developed. Work by several groups linked the silencing of the $p16^{INK4a}$ gene by CpG methylation in the promoter to cancer (Cairns, Polascik et al. 1995, Gonzalez-Zulueta, Shibata et al. 1995, Herman, Merlo et al. 1995). Since then, multiple examples of cancer-related epigenetic defects in the literature have been described including hypermethylation of *GSTP1* in prostate cancer (Cairns, Esteller et al. 2001, Lin, Tascilar et al. 2001), hypermethylation of *DAPK* in bladder cancer (Catto, Azzouzi et al. 2005, Jarmalaite, Andrekute et al. 2010) and promoter methylation of $p16^{NK4a}$, *RASSF1A*, and *APC* in early stage non-small cell lung cancer (Brock, Hooker et al. 2008). Interestingly, analysis of over 600 different tumor types by Esteller et al. revealed that 80% of these had hypermethylation of a CpG island upstream at least one TSG, with 5-10% having this pattern at three or more TSG loci (Esteller, Corn et al. 2001). Breast cancer is no exception, with findings of *RASSF1A* promoter methylation in 80-95% of cases and inactivated $p16^{NK4a}$ detected in approximately 30% of high risk females (Yeo, Wong et al. 2005, Bean, Bryson et al. 2007). In fact, there have been reports of silencing of several other TSGs in breast cancer, including *CCND2, RARB, APC* and *CDH1* (Evron, Umbricht et al. 2001, Jin, Tamura et al. 2001, Farias, Arapshian et al. 2002, Sebova, Zmetakova et al. 2011). These effects are found to occur early in the progression of breast cancer and can be used to distinguish tumorigenic tissue from normal samples. Interestingly, promoter methylation of CpG islands is found to increase as breast cells progress from a normal state to atypical ductal hyperplasia to ductal carcinoma *in situ*. The invasive breast tumor, however, does not acquire significantly greater promoter methylation in comparison to the *in situ* carcinoma, suggesting that epigenetic dysfunction is among the earlier steps leading to cellular malignancy (Park, Kwon et al. 2011).

Defects in post translational modifications of histones are also common in cancer. For instance, histone deacetylases (HDACs) can become overexpressed in certain tumors, including

those of colon, gastric and breast origin, where they can act to remove acetyl marks from histones and promote gene silencing (Rosato and Grant 2005, Zhang, Yamashita et al. 2005, Wilson, Byun et al. 2006). The importance of maintaining acetylated histones on growth control is reflected by the strong correlation between depleted H4K16 acetylation in the genome and cancer progression, for example (Fraga, Ballestar et al. 2005). This can lead to pro-tumorigenic changes given that some of the silenced targets are TSGs.

While there is a clear role for epigenetic defects in cancer, there is also strong evidence that targeting these changes can improve cancer survival. What is particularly intriguing about epigenetic defects in cancer, in comparison to genetic alterations, is the fact that epigenetic changes are reversible and can therefore be targeted with drugs.

1.10 | Epigenetics in the clinic: successes and future potential

1.10.1 | DNA methylation inhibitors

The exploitation of epigenetics for therapy has had much success in clinical trials. Among the first epigenetic drugs to hit the market were the the DNA methylation inhibitors 5-azacytidine (5-azaC) and its deoxy analog 5-deoxycytidine (5-azaCdR) (Issa and Kantarjian 2009). These nucleoside analogs are known to become integrated in the DNA where they promote chromosome breakage and consequently reduce the rate of cell growth (Karon and Benedict 1972, Viegas-Péquignot and Dutrillaux 1976). In addition, these compounds were also found to irreversibly bind Dnmts and inhibit their function (Creusot, Acs et al. 1982, Taylor and Jones 1982). Consequently, use of these drugs generate a hypomethylated state in the cell which is believed to induce the formation of transcriptionally active chromatin and to restore expression of TSGs (Ghoshal, Datta et al. 2005). This was confirmed by various studies that have tested the effect of Dnmt1 depletion on gene expression and cell proliferation. These have revealed a restoration of TSG expression and the regulation of cancer cell growth *in vitro* (Suzuki, Sunaga et al. 2004, Oridate and Lotan 2005, Foltz, Yoon et al. 2009). 5-azaC and 5-azaCdR, through Dnmt1 inhibition, likely work in this manner to regulate cell cycle progression in addition to their role in inducing chromosomal instability. These drugs are currently in use to treat myelodysplastic syndromes, having provided a 24% increase in survival after 2 years, compared

to the standard treatment (Fenaux, Mufti et al. 2009) as well as for acute myelogenous leukemia and chronic myelomonocytic leukemia (Fahy, Jeltsch et al. 2012). Phase II clinical trials are also ongoing to assess the efficacy of these drugs against solid tumors including melanoma, ovarian and prostate cancer (Gros, Fahy et al. 2012). While therapeutically effective, 5-azaC and 5-azaCdR have poor bioavailabities, are chemically unstable and are rather toxic, mainly due their integration into the DNA (Gravina, Festuccia et al. 2010). Consequently, new efforts have been focused on developing more stable and specific inhibitors against Dnmt1. These drugs are currently in the early stages of clinical trials (Fahy, Jeltsch et al. 2012) for which we eagerly await the results.

1.10.2 | Histone deacetylase inhibitors

The histone deacetylase inhibitors (HDACi), like inhibitors of DNA methylation, are believed to be therapeutically relevant due to the restoration of loose chromatin and induction of changes in transcription, among other things (Kim and Bae 2011). Interestingly, it has been proposed that the expression of 20% of known genes is affected by HDACi. Of these genes, half become upregulated in response to the drug (Minucci and Pelicci 2006). This can have implications in various processes in the cell. For instance, HDACi have been involved in the restoration of TSG expression including that of *p21*, promoting the growth control of cells (Richon, Sandhoff et al. 2000, Sandor, Senderowicz et al. 2000). They have also been speculated to be involved in apoptosis by inducing the expression of pro-apoptotic factors such as *BIM* and *BMF* (Zhang, Adachi et al. 2005, Zhao, Tan et al. 2005). In addition to having a role in transcriptional regulation, HDACi have been proposed to increase the sensitivity of cells to DNA damaging agents through the loosening of chromatin. They have also been proposed to disrupt important protein-protein interactions. For instance, it is believed that HDACi can inhibit the interaction between the DNA-damage response protein Ku70 with the pro-apoptotic protein Bax, ultimately allowing the process of programmed cell death to ensue (Minucci and Pelicci 2006). Together, these effects coordinately mediate an anti-cancer effect in the cell.

Given the multi-pronged attack described for this class of drug, it comes as no surprise that the HDACi vorinostat and romidepsin have been approved for treatment of cutaneous T-cell lymphoma (CTCL) patients in 2006 and 2009 respectively (Marks and Breslow 2007, Monneret

2007, Campas-Moya 2009). With 30% improvement in the response rate of CTCL patients treated with vorinostat and clinical benefits observed for otherwise non responding patients treated with romidepsin, there has been an increase in excitement surrounding epigenetic drugs in the clinic (Olsen, Kim et al. 2007, Piekarz, Frye et al. 2009). As a result, several new clinical trials testing the efficacy of HDACi are underway. HDACi such as mocetinostat and panobinostat are currently under study for the treatment of relapsed classical Hodgkin's lymphoma and high grade glioma respectively (Younes, Oki et al. 2011, Drappatz, Lee et al. 2012). At the same time, other trials have focused on the efficacy of HDACi in combination with current cancer treatments. Already, such combinations as vorinostat/paclitaxel/bevacizumab have found to have a 55% response rate in advanced breast cancer and a vorinostat/tamoxifen combination was found to have a 40% clinical benefit rate in advanced ER positive breast cancers (Gray, Bhattacharya et al. 2009, Ramaswamy, Fiskus et al. 2012). Clearly there is huge potential for these drugs in cancer.

1.10.3 | Histone methylase inhibitors

In addition to targeting histone acetylation, other drugs are being designed to regulate the levels of histone methylation. In particular, these drugs are involved in the inhibition of histone methyltransferases including the polycomb repressor protein EZH2, responsible for the addition of the H3K27me3 mark to chromatin. Upregulation of EZH2 is known to be involved in breast and prostate cancer progression and it is a marker of poor prognosis for patients (Varambally, Dhanasekaran et al. 2002, Kleer, Cao et al. 2003, Takawa, Masuda et al. 2011). Mechanistically, this is believed to result from the generation of transcriptionally repressive chromatin through widespread addition of H3K27me3 that may silence TSGs. This has been shown to be the case for the polycomb repressor protein Bmi-1 whose expression is inversely linked to that of the TSG $p16^{INK4a}$ (Jacobs, Kieboom et al. 1999). Pre-clinical studies testing the effect of EZH2 inhibitors have discovered a therapeutic benefit for these drugs in the treatment of mutant lymphomas (Knutson, Wigle et al. 2012, McCabe, Ott et al. 2012). These drugs, however, have only recently entered phase I clinical trials in which we anticipate positive results in the near future.

1.10.4 | Inhibitors of protein interactions with chromatin

In addition to targeting the enzymatic activity of DNA or histone modifying enzymes, new avenues of research are also focused on disrupting the interaction of these enzymes with the chromatin. Specifically, a new cell-permeable drug named JQ1 acts in this manner to bind bromodomains, responsible for mediating protein binding to acetylated lysines. As such, JQ1 disrupts the association between proteins and their targets on the DNA (Filippakopoulos, Qi et al. 2010). Among the proteins affected by the drug is the Brd4 protein that binds acetylated histones through its bromodomains. Appropriate regulation of this factor is important given that a translocation between the BRD4 bromodomains and the NUT protein has been described to occur in NUT midline carcinoma (NMC) (French, Ramirez et al. 2007). The localization of this protein is important given its role in recruiting P-TEFb, a transcriptional elongation factor responsible for inducing the expression of cell cycle progression genes (Yang, He et al. 2008). Interestingly, JQ1 disrupts the interaction of Brd4 with chromatin. At the same time, this drug has proven to be effective in xenograft models of NMC (Filippakopoulos, Qi et al. 2010). This result reveals a promising future for molecules that disrupt protein/histone interactions.

Clearly, there is great potential for epigenetic drugs in cancer treatment and many new drugs are entering clinical trials. Still, this branch of research is in its early stages and much work is to be done in the field. The purpose of this work is to define novel mechanisms through which TSGs become silenced due to shortcomings of current models. Hopefully, obtaining insight into the mechanism whereby TSGs become silenced will reveal new therapeutic targets.

1.11 | Ctcf

The work from our lab has indicated a key epigenetic regulatory protein termed ‘Ctcf’ is critical for maintaining expression of TSGs. Ctcf binding at TSGs is often lost in cancer cells where TSGs are epigenetically silenced (Witcher and Emerson 2009, Recillas-Targa, de la Rosa-Velázquez et al. 2011). We predict that understanding the mechanism whereby Ctcf binding is lost from TSGs in cancer will provide new therapeutic targets that aim to restore TSG expression.

Ctcf is a ubiquitously expressed and highly conserved eleven zinc-finger DNA binding protein often considered as the master regulator of the genome (Figure I). Throughout evolution, Ctcf has remained largely unchanged, given the near 100% sequence conservation between the mouse, chicken and human genomes (Ohlsson, Renkawitz et al. 2001). Sequence conservation can also be detected in lower organisms including flies (Moon, Filippova et al. 2005) and some nematodes (Heger, Marin et al. 2009), stressing the importance of Ctcf in regulating normal cell biology. Initially, Ctcf was named for binding the CCCTC motifs near the c-myc promoter in chickens (Lobanenkov, Nicolas et al. 1990). Since then, Ctcf binding has been found to extend over a 50-60 bp sequence that can diverge widely between sites (Ohlsson, Renkawitz et al. 2001). This can be further refined to a 12-base pair consensus sequence (5'-CCGCTAGGGGGC-3'), recognized specifically by zinc fingers 4 to 8, that encompasses much of the Ctcf sites bound throughout the genome (Renda, Baglivo et al. 2007). The variability in the extended sequence is likely due to the involvement of the additional zinc fingers for binding (Ohlsson, Renkawitz et al. 2001).

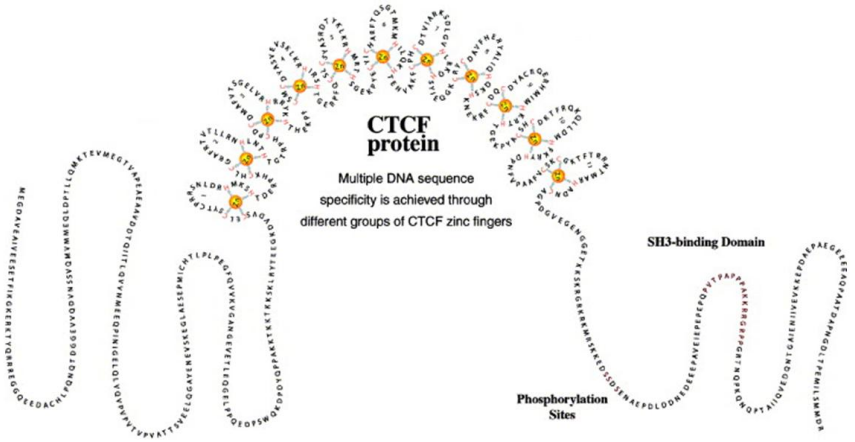

Figure I. Structure of the CCCTC transcription factor, Ctcf (Klenova, Morse et al. 2002). Ctcf is a transcription factor possessing an N- and C-terminal domain, with a centrally located DNA binding domain. The central domain is composed of 11-zing fingers that can be used in several combinations to bind DNA. Ultimately, different combinations of zinc finger binding throughout the genome can account for the promiscuity of Ctcf binding to DNA.

ChIP-seq studies have been vital in defining a consensus sequence for Ctcf binding, although discrepancies between different laboratories can be noted. For instance, analysis of

IMR90 human fibroblasts revealed 13,804 CTCF-binding sites throughout the genome. Among these, 46% were found to be intergenic, 22% intronic, 12% exonic, and 20% within 2.5 kb of promoters (Kim, Abdullaev et al. 2007). Characterization of Ctcf binding sites in resting CD4+ T cells revealed 20,262 sites with 41% of these residing within intergenic regions, 31% within transcribed regions and 28% within 2 kb of the transcription start site (TSS) (Barski, Cuddapah et al. 2007, Jothi, Cuddapah et al. 2008). Still, other ChIP-seq studies detected as many as 39,609 Ctcf-binding sites in mouse embryonic stem (ES) cells, 19,308 in HeLa cells and 19,572 in Jurkat cells (Chen, Xu et al. 2008, Cuddapah, Jothi et al. 2009). Whether these varying findings are due to lineage-specific differences or whether these effects are the result of differences in experimental procedures remains unknown, although the reality is likely a mix between both alternatives.

With numerous binding sites having been described throughout the genome, it can be speculated that Ctcf plays a vital role in normal cellular biology. Indeed, homozygous deletions of Ctcf in genetically engineered mice results in early embryonic lethality (Moore, Rabaia et al. 2012). This phenotype, in combination with the high sequence homology of the Ctcf gene retained across species barriers, is a testament to the importance of this factor in cellular functioning. RNAi depletion experiments revealed that Ctcf is involved in slowing the rate of cellular proliferation and in mediating cellular differentiation. Overexpression studies implicated Ctcf in apoptosis as well (Torrano, Chernukhin et al. 2005). Indeed, missense mutations in Ctcf that interfere with certain aspects of its biological function have been detected in breast, prostate and Wilm's tumors (Filippova, Qi et al. 2002). Specifically, these mutations were found to disrupt the binding of Ctcf to certain target sites. Ultimately, these changes can have effects on Ctcf-regulated promoter activation and repression, gene silencing, imprinting as well as chromatin insulation (Ohlsson, Renkawitz et al. 2001). These roles are further described below to paint a clearer picture of the effect of Ctcf in biology and in disease, in particular, cancer.

The ability of Ctcf to act as an insulator has been one of the most intriguing characteristics of this protein to date. This may be due to the fact that thus far, Ctcf is the only known protein in vertebrates to mediate this function (Dunn and Davie 2003). The ability of Ctcf to act as an insulator implies that it acts to prevent the action of enhancers or repressors on distal gene promoters. This role is consistent with reports that Ctcf binding can be detected near regions of genes that are transcriptionally co-regulated (Kim, Abdullaev et al. 2007, Xie,

Mikkelsen et al. 2007). However, Ctcf binding in between genes was linked to opposite regulation of these (Xie, Mikkelsen et al. 2007).

The classic example of Ctcf insulator activity is the control of the H19/Igf2 locus (Figure II). Regulation at this site involves the differentially methylated imprinted control region (ICR), the sequence of DNA to which Ctcf binds. Under circumstances where this site is unmethylated, as is the case for the maternally inherited locus, Ctcf can bind the ICR and act as a barrier between Igf2 and its enhancer (Bell and Felsenfeld 2000, Hark, Schoenherr et al. 2000, Szabó, Tang et al. 2000). This prevents transcription of Igf2, and leaves the enhancer free to promote the expression of the proximal H19 gene instead. Methylation present in the paternally inherited ICR disrupts Ctcf binding, allowing the enhancer to preferentially activate Igf2 transcription. Experiments where the ICR was mutated in the maternal allele resulted in a loss of Ctcf binding and permitted the broad accumulation of DNA methylation in mice (Engel, Thorvaldsen et al. 2006). This promoted a situation similar to that in the paternal allele where the loss of enhancer blocking activity leads to the expression of Igf2 (Szabó, Tang et al. 2004). The loss of Ctcf binding was also associated to a reduction of the H3K9ac, H3K4me2, and H3K4me3 marks at the H19 promoter with a concordant loss of H3K27me3 at the *Igf2* promoter (Han, Lee et al. 2008). This suggests that Ctcf binding is important for maintaining proper epigenetic marks, at least at this locus.

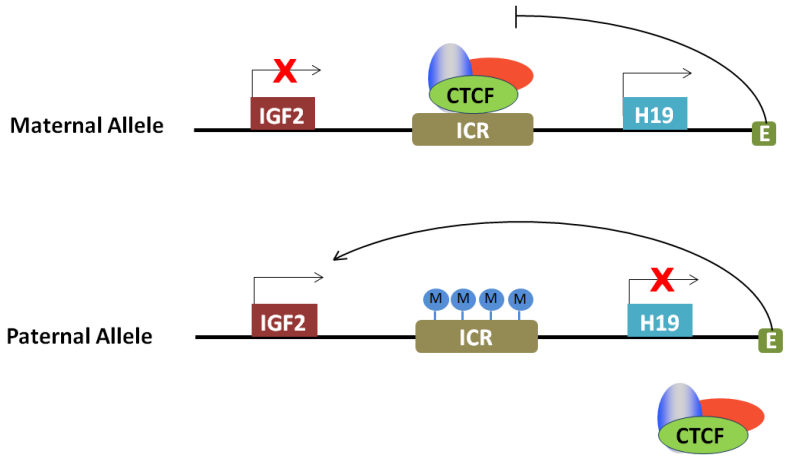

Figure II. The H19/IGF2 locus gene expression is differentially regulated in the maternal and paternal alleles by Ctcf. In the maternally inherited allele, Ctcf can bind the imprinted control region (ICR) and act as an insulator between the enhancer (E) and the IGF2 promoter. In the paternal allele, however, the imprinted control region is methylated (M), ultimately preventing the binding of Ctcf to the DNA. As such, the enhancer is free to induce high IGF2 expression, essentially shutting off H19 expression.

Similar findings were reported for Ctcf insulation at the chicken β-globin locus, a structurally and functionally conserved site among vertebrate species including mice and humans (Hardison, Slightom et al. 1997). This locus, in particular, is juxtaposed to a group of differentially regulated genes. At the 5' end of the chicken β-globin locus resides a pre-erythroid-specific folate receptor gene and at the 3' end resides a pair of olfactory receptor genes (Bulger, Von Doorninck et al. 1999, Prioleau, Nony et al. 1999). Consequently, important control mechanisms must be in place to ensure regulated and timely expression of these factors. This specific regulatory role is believed to be mediated by the 5' and 3' DNase I-hypersensitive sites (HSs) flanking the β-globin locus. These sites have roles in gene insulation, and require Ctcf binding for functioning (Saitoh, Bell et al. 2000). Interestingly, within the chicken locus, these sites mark abrupt boundaries between loose and dense chromatin (Hebbes, Clayton et al. 1994, Litt, Simpson et al. 2001). The functional significance of these sites within the human and mouse locus still remains unclear as mutations of these sites such as the 3' hypersensitive site was not found to induce spurious gene transcription in mouse cells (Splinter, Heath et al. 2006). Nevertheless, it is apparent that Ctcf is important in the regulation of gene expression at certain sites throughout the genome.

1.11.1 | Ctcf chromatin loops

One of the mechanisms through which Ctcf is thought to regulate the function of enhancers is through chromosome looping in the cell (Phillips and Corces 2009). Chromosome looping is thought to be important for bringing distal regions of the DNA into close proximity for appropriate gene regulation (Ptashne 1986). Ctcf binding is known to occur at various sites throughout the genome and the bridging of these sites is believed to form chromatin loops. This is supported by yeast-two hybrid analysis showing that Ctcf can dimerize *in vivo* (Yusufzai, Tagami et al. 2004).

The use of 3C technology for analysis of the H19/Igf2 locus has detected loops within the DNA at this site (Murrell, Heeson et al. 2004, Kurukuti, Tiwari et al. 2006). This higher order structure of DNA, formed specifically on the maternally inherited chromosome where Ctcf binds, promotes the recruitment of the Polycomb Repressor Complex 2, through interaction with one of its components, Suz12. In turn, this complex mediates silencing of the Igf2 locus on this

allele through the addition of H3K27me3 (Li, Hu et al. 2008). The addition of this repressive histone mark is critical for Igf2 silencing, given that chromatin looping alone, in a Suz12 deficient background, was found not to be sufficient to mediate gene repression (Han, Lee et al. 2008).

Similarly, chromosome looping was found at the mouse β-globin locus. In particular, it has been proposed that Ctcf is involved in forming a 'hub', a 3D aggregate of hypersensitive sites, where other factors coordinately bind to regulate gene functioning (Tolhuis, Palstra et al. 2002). Interestingly, this effect was cell-type specific, with cells of erythroid lineage exhibiting long-range chromatin interactions at this site but not brain cells (Tolhuis, Palstra et al. 2002, Palstra, Tolhuis et al. 2003, Splinter, Heath et al. 2007). Disruption of the Ctcf binding sites both upstream and downstream the β-globin gene, while negatively impacting the formation of chromatin loops, was not found to alter gene expression at this locus (Splinter, Heath et al. 2006). Thus, perhaps at some sites throughout the genome, Ctcf possesses a structural role that is dispensable for transcription control. This highlights the selectivity of Ctcf in regulation transcription throughout the cell.

1.11.2 | Ctcf as a boundary forming factor

In addition to its insulator function, ChIP experiments and knockdown studies indicate that Ctcf plays an import role in the maintenance of chromatin boundaries (Cuddapah, Jothi et al. 2009, Witcher and Emerson 2009). Repressive heterochromatin is the default state and, unless constrained, will spread passively throughout a chromosome (Talbert and Henikoff 2006). Interestingly, it has been reported that islands of euchromatin embedded within large expanses of heterochromatin are highly enriched for Ctcf binding (Wen, Wu et al. 2012). This correlates with findings that that a certain proportion of Ctcf binding sites demarcate the boundaries between active and repressive chromatin, delineating regions enriched with the activating H2AK5Ac mark from regions of the repressive H3K27me3 mark (Cuddapah, Jothi et al. 2009). In fact, it has been reported that 74% of genes are flanked in their entirety by Ctcf binding sites, including such genes as *PPP5C, PAK4* and *GNAS* (Kim, Abdullaev et al. 2007). Taken together, this strengthens the notion that Ctcf acts as a boundary, preventing the passive spread of heterochromatin over gene loci.

1.11.3 | Ctcf effect on transcription

The roles of Ctcf in enhancer blocking and in maintaining chromatin boundaries entails that this factor can have varying effects on transcription depending on the context. For instance, given its effect in enhancer blocking, it comes as no surprise that Ctcf has been linked to gene silencing. Indeed, initial studies characterized Ctcf as being a repressor protein, as it was found to inhibit the expression of c-myc (Klenova, Nicolas et al. 1993, Filippova, Fagerlie et al. 1996). Likewise, another study found that Ctcf could also repress thyroid hormone response element (TRE) gene transcription (Awad, Bigler et al. 1999). The role of Ctcf in gene repression is further highlighted by its role in X-chromosome inactivation (Chao, Huynh et al. 2002). While the role of Ctcf in enhancer blocking can result in gene repression, its role in maintaining chromatin boundaries is more typically associated with a positive role for Ctcf in gene expression. In fact, throughout the genome, Ctcf binding has been characterized as occurring more frequently upstream active CpG promoter regions (Vavouri and Lehner 2012). Moreover, Ctcf binding sites show a strong positive correlation with histone activating marks H4K20me1, H3K9me1, H3K9ac and H3K4me3 in various cell types and were most often linked to unmethylated CpG islands (Chen, Tian et al. 2012). This is consistent with a positive role for Ctcf in gene expression at certain sites. In accordance with these results, Ctcf has been linked to expression of the TSG Amyloid β-Protein Precursor (Vostrov and Quitschke 1997). The role of Ctcf in maintaining TSG expression has also been described in other cases. In particular, decreased expression of Ctcf in cell culture models resulted in the silencing of TSGs like Brca1 and Rb through excessive methylation (Butcher and Rodenhiser 2007, Dávalos-Salas, Furlan-Magaril et al. 2011). Taken together, the data seems to support a differential role for Ctcf in transcriptional regulation. Thus, the control of Ctcf levels and binding throughout the genome may be an important level of regulation that may be affected in diseased states such as cancer.

1.11.4 | Ctcf post translational modifications

While the effect of Ctcf on transcription can be a function of the levels of protein within the cell, there is also a role for Ctcf post-translational modifications in gene regulation. Here, we describe some of the known modifications that can modulate Ctcf activity. These changes may

become altered in malignant cells and if so, could potentially be easily reversible targets for therapy.

1.11.4.1 | Ctcf phosphorylation

Phosphorylation is one of the best characterized post-translational modifications in the cell, often involved in signal transduction cascades leading to downstream transcriptional effects. Depending on the context, phosphorylation can positively or negatively affect the activity of enzymes, the DNA binding of transcription factors or control the cellular compartmentalization of cells (Bohmann 1990, Hunter and Karin 1992). One of the ubiquitous proteins in the cell that mediate the addition of this post-translational modification is the Casein Kinase II (CKII). Specifically, this protein phosphorylates serine or threonine residues found within the sequence S/TXXDE (where the X represents any non-basic amino acid) (Litchfield 2003). Interestingly, this domain can be detected at several sites within the C-terminal region of Ctcf. Phosphorylation at these sites is associated with a change from a transcriptionally repressive function for Ctcf to an activating one (El-Kady and Klenova 2005). Thus, the changes in phosphorylation states of Ctcf can be important in regulating gene expression in the cell. To date, however, there has not been any concrete evidence for deregulation of Ctcf phosphorylation in cancer. For this reason, there has not been any further investigation into the modulation of Ctcf phosphorylation for therapy.

1.11.4.2 | Ctcf SUMOylation

A relatively new post-translational modification making its way into the literature is that of SUMOylation. While considered part of the ubiquitin-like family of proteins, the addition of SUMO does not mediate protein degradation (Kerscher, Felberbaum et al. 2006). Instead, this covalent post-translational modification has been implicated in various roles including transcriptional regulation, chromatin structure, and DNA repair. Indeed, Ctcf has been shown to be modified by SUMOylation at a site in its N-terminal and C-terminal domain. This change has been shown to promote repression of the *c-MYC* gene by Ctcf (MacPherson, Beatty et al. 2009).

While a potentially interesting target for therapy, the effect of SUMOylation in cancer has not been well characterized and still requires much research.

1.11.4.3 | Ctcf PARylation

In addition to being modified by phosphorylation and SUMOylation, Ctcf has been shown to be modified by poly(ADP-ribosyl)ation (PARylation) in its N-terminal domain (Yu, Ginjala et al. 2004). This post-translational modification is added by the poly(ADP-ribose) polymerase enzyme (Parp) onto glutamate, aspartate and lysine residues and is removed by the poly(ADP-ribose) glycohydrolase enzyme (Parg). Non-covalent PAR-binding can also occur through a specific domain characterized by interspersed basic and hydrophobic residues , the PBZ motif or via the macrodomains (Krishnakumar and Kraus 2010). Interestingly, unlike Ctcf phosphorylation or SUMOylation, decreased poly(ADP-ribosyl)ation of Ctcf has associated with breast cancer phenotype and cell proliferation. In particular, the presence of dePARylated Ctcf has been detected in breast tumors and this was associated with tumor stage (Docquier, Kita et al. 2009). Specifically, it was illustrated that a lower molecular weight form of Ctcf (corresponding to the dePARylated protein) was present in a large percentage of tumorigenic breast tissue, while only the higher molecular weight form of Ctcf (PARylated Ctcf) was detected in normal breast tissue. Culturing normal cells *in vitro* reveals a shift from the PARylated form of Ctcf to the unPARylated one after a week. This suggests that the unPARylated form of Ctcf is associated to an increased proliferative capacity of cells. Alternatively, restoring growth control through the inhibition of the cell cycle restored the expression of PARylated Ctcf (Docquier, Kita et al. 2009). Clearly, there is an important link between dePARylation of Ctcf and cell cycle progression. As a result, regulating the levels of PARylation in the cell may be an interesting new therapeutic approach for cancer where growth control is lost.

Among the functional properties of PARylation is its role in maintaining Ctcf insulation, initially discovered at the H19/Igf2 locus. Interestingly, ChIP analyses of Ctcf or PAR revealed similarities in their binding sites throughout the genome (Yu, Ginjala et al. 2004). These results allude to the possibility that inhibition of Ctcf PARylation may result in altered regulation of numerous target genes through the aberrant actions of distal regulatory regions. Likewise, Ctcf

boundary function has also been described to depend on covalent modification of the protein by PARylation. Loss of this modification has shown to suppress the expression of TSGs like *p16*^{INK4a} and *RASSF1A* by the spread of repressive histone marks throughout the gene loci (Witcher and Emerson 2009). This alludes to a role for Ctcf and PARylation in establishing proper gene expression profiles, including that of TSGs which are important in maintaining cell growth control. The role of Ctcf and PARylation was further characterized in L929 fibroblasts where it was demonstrated that exogenous Ctcf expression increased nuclear PARylation by direct stimulation of Parp-1. These changes were linked to a reduction in cell proliferation. The additional Ctcf introduced to the system was found to inhibit Dnmt1 activity, reducing levels of DNA methylation. Interestingly, this was not the case in Parp-1 knockout cells, showing a requirement for Parp-1 to mediate Dnmt1 inhibition (Guastafierro, Cecchinelli et al. 2008). Together, the results support the notion that Ctcf is involved in maintaining active chromatin, in this case, through the reduction of DNA methylation. Overall, these findings highlight the role of Ctcf and PARylation in chromatin decondensation and illustrate how these factors can induce TSG expression and regulate the growth of cells.

Given the importance of PARylation in growth control described above, we now turn to the widely popular Parp inhibitors that have generated much excitement in the clinic in recent years. Having now characterized the role of PARylation in slowing cell growth and maintaining TSG expression, it seems counterintuitive that drugs that target Parp could have a beneficial effect for cancer therapy. However, there have been many reports of success of Parp inhibitors in oncology. Here below, we provide an extensive review of the uses of Parp inhibitors in the clinic, as well as their limitations. The following is a peer-reviewed and published text with slight modifications for the integration into this thesis (Lovato, Panasci et al. 2012).

1.12 | The clinical landscape of Parp inhibitors as anti-cancer therapeutics

Much of the success of Parp inhibitors has been achieved in cells with mutations in the *Brca1/2* genes. These mutations are present in approximately 10-20% of triple-negative tumors (Gonzalez-Angulo, Timms et al. 2011, Pern, Bogdanova et al. 2012, Phuah, Looi et al. 2012). Brca1/2 proteins are an integral part of the homologous-recombination (HR) mediated DNA damage repair process. When mutated, the repair of double-strand breaks by HR is

compromised. Mutations within the *Brca* genes are known to confer a 50-80% lifetime risk of breast cancer and a 20-40% lifetime risk of ovarian cancer for female carriers (Ford, Easton et al. 1998). An exciting new development in the field of cancer therapy is the exploitation of this defect using Parp inhibitors to generate synthetic lethality. Parp inhibitors have been used successfully to achieve clinical responses in patients with Brca mutated breast or ovarian cancers (Fong, Boss et al. 2009, Audeh, Carmichael et al. 2010, Tutt, Robson et al. 2010). Notably, the adverse effects of Parp inhibitors seen in clinical trials are quite mild with few signs that non-tumorigenic tissue is targeted by these drugs.

The role of Parp in the DNA damage repair response is multifaceted and already well-reviewed (Aly and Ganesan 2011, Helleday 2011). It is generally suggested throughout the scientific literature that Parp inhibitors generate an accumulation of single-strand DNA breaks in Brca mutated cells, which are subsequently processed to double-strand breaks during replication. However, the precise mechanism whereby Brca mutations and Parp inhibition combine to create a synthetic lethal effect remains somewhat controversial, with multiple mechanisms proposed (Helleday 2011). Nonetheless, it seems clear that Parp inhibitors lead to stalled replication forks and an accumulation of DNA damage, particularly cytotoxic in cells with a mutant Brca background.

The clinical efficacy of Parp inhibitors in triple-negative breast tumors bearing Brca mutations has sparked interest to initiate new clinical trials in triple-negative patients having wild-type Brca with the expectation that combining chemotherapy with Parp inhibitors will enhance the outcomes of currently utilized therapies. While the results from a few phase one trials are encouraging, to date, most have been met with limited success. Preliminary data from a cohort of 86 triple-negative patients co-treated with the Parp inhibitor Bsi-201 (iniparib) and gemcitabine showed improved overall survival (O'Shaughnessy, Osborne et al. 2009). Similarly, co-treatment of a 123 triple-negative patient cohort with gemcitabine and iniparib improved overall survival from 7.7 to 12.3 months (O'Shaughnessy, Osborne et al. 2011). But a larger phase III trial involving 519 women showed cotreatment of cytotoxic agents with the Parp inhibitor Iniparib was associated with disease progression in most cases (O'Shaughnessy 2011). However, results from these trials should be taken with caution as recent data suggests Parp is not the primary target of iniparib (Liu, Shi et al. 2012). Another phase II trial looking at the efficacy of the Parp inhibitor olaparib in breast cancer patients failed to show significant clinical

responses (Gelmon, Tischkowitz et al. 2011). Encouragingly, in this same study, a cohort of patients with ovarian cancers did demonstrate partial responses to olaparib regardless of Brca status.

Overall, the early indicators from trials involving Parp inhibitors for triple-negative breast cancer show partial, but not complete responses. Encouragingly, there are clearly patients who do respond to these therapies. Recent research has identified a subset of triple-negative tumors that have an increased likelihood to respond to Parp inhibition. Defective proteins in the homologous recombination repair system, or epigenetically silenced Brca, amongst other defects, contribute to a molecular pathology that is not unlike tumors bearing Brca mutations. These tumor properties are defined as having "Brcaness" or being "Brca-like" (Turner, Tutt et al. 2004, Ratner, Sartorelli et al. 2012). This concept has led to the hypothesis that tumors with features of Brcaness may respond to Parp inhibition. The concept of Brcaness has been used in retrospective study to predict response to platinum-based therapies in 8 of 10 patients (Konstantinopoulos, Spentzos et al. 2010). But a recent study analyzing data from 101 patients receiving adjuvant cyclophosphamide-based chemotherapy showed Brcaness could not predict differences in patient survival (Oonk, van Rijn et al. 2012). However, it still remains to be determined if pathological features of Brcaness may be a more powerful predictor of sensitivity to Parp inhbitors than conventional chemotherapy.

Current literature suggests several hypotheses to predict sensitivity to Parp inhibition in subsets of triple-negative tumors, but there is a lack of evidence from *in vitro* and mouse studies suggesting that established triple-negative cell lines are sensitive to clinically relevant Parp inhibitors or Parp-1 knock down. In fact, unpublished data from our lab and another recent report show very high concentrations of commonly used Parp inhibitors are needed to suppress the growth of triple-negative cell lines *in vitro* (Chuang, Kapuriya et al. 2012). The micromolar concentrations of inhibitors needed to suppress proliferation is likely well beyond those required to block Parp activity (Bryant, Schultz et al. 2005, Liu, Shi et al. 2008), and may reflect secondary effects of these inhibitors.

Beyond breast cancer, early phase clinical trials with Parp inhibitors in combination with standard chemotherapy have been met with either partial responses, or a lack of clinically relevant responses in multiple types of solid tumors (Plummer, Jones et al. 2008, Khan, Gore et al. 2011, Kummar, Chen et al. 2011, Kummar, Ji et al. 2012, Rajan, Carter et al. 2012). We

propose that activities of Parp, being targeted by inhibitors beyond those in the DNA damage repair process, account for their limited success in a wild-type Brca background (fig.1). Specifically, we hypothesize that targeting Parp will impair its role in regulating the expression of tumor suppressor genes, thereby generating unwanted consequences. However, we further propose that understanding the mechanisms whereby Parp activates transcription may be used to predict new, more potent therapeutic approaches.

1.13 | Poly(ADP)polymerases, more than DNA repair enzymes (adapted from Lovato et al., 2012, Frontiers in Pharmacology of anti-cancer drugs).

The Parp family of enzymes encompasses multiple proteins (up to 17) of varying degrees of homology (with the main conservation residing in the Parp catalytic domain), all of whom use NAD+ as a substrate to catalyze the addition of ADP-ribose moieties onto target proteins (Kim, Zhang et al. 2005). Among the Parp proteins, only Parp-1 and Parp-2 build "poly" ADP-ribose polymers. The other family members, including Parp-3, are capable of adding only a monomeric ADP-ribose to proteins. Of Parp-1 and 2, Parp-1 has been more extensively studied and will therefore be the focus of this review. Under basal conditions, Parp-1 is active, but modifies relatively few target proteins compared to conditions of cellular stress and after DNA damage (Gagne, Isabelle et al. 2008, Witcher and Emerson 2009, Gagne, Pic et al. 2012). Parp-1 recognizes and binds specific DNA secondary structures commonly associated with damaged DNA including single-strand DNA, double-strand breaks, and crossovers. Upon binding, its enzymatic activity is allosterically triggered(D'Amours, Desnoyers et al. 1999, Kun, Kirsten et al. 2002). This particular mechanism of regulation allows localized activation of Parp-1 for targeted repair of DNA damage. However, numerous studies have also demonstrated that Parp-1 is recruited to chromatin, which acts as an on-switch for its enzymatic activity in the absence of DNA damaging agents (Poirier, de Murcia et al. 1982, Ding and Smulson 1994, Kim, Mauro et al. 2004, Lonskaya, Potaman et al. 2005, Pinnola, Naumova et al. 2007, Wacker, Ruhl et al. 2007).

Parp-1 associates with the chromatin of promoter regions in a significant proportion of actively transcribed genes throughout the genome (Krishnakumar and Kraus 2010, Zhang, Berrocal et al. 2012) and copurifies biochemically with RNA Pol II (Slattery, Dignam et al.

1983). Consistent with this, multiple studies utilizing Parp-1 depleted cells have substantiated an activating role for Parp-1 in gene regulation (Ziegler and Oei 2001, Ogino, Nozaki et al. 2007, Krishnakumar, Gamble et al. 2008, Okada, Inoue et al. 2008, Frizzell, Gamble et al. 2009).

How does Parp modulate gene expression? It does so through a multi-pronged approach, with individual actions cooperating to fine tune the transcriptional process (Figure III). Parp-1 regulates transcription minimally through (1) the alteration of chromatin structure (2) the control of DNA and histone methylation status (3) the recruitment and maintenance of transcription factors to promoter regions and (4) acting as a transcriptional coregulator.

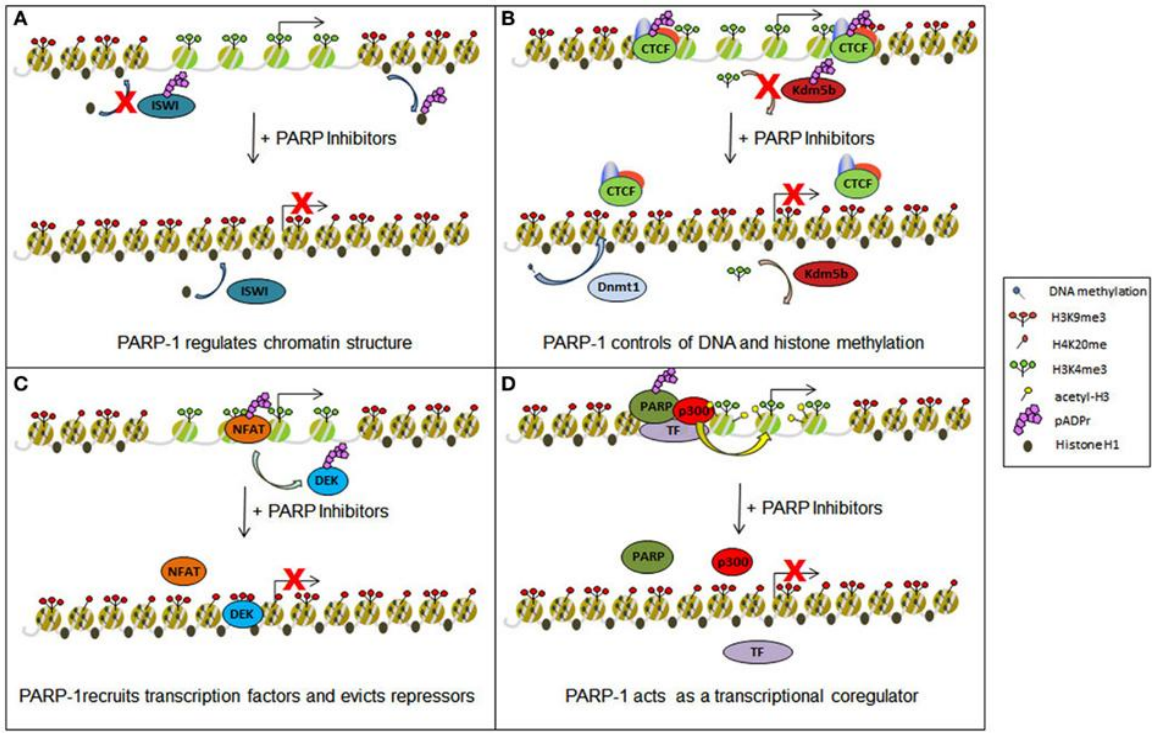

Figure III. Epigenetic and transcriptional impact of Parp inhibitors. Parp inhibitors may contribute to epigenetic and transcriptional deregulation in cells through several different mechanisms. **A.** The drug-induced spread of heterochromatin may result from the release of the protein Iswi from inhibition, promoting histone H1 integration into chromatin, and through the prevention of histone H1 removal from chromatin by direct PARylation. **B.** Chromatin boundaries normally maintained by PARylated Ctcf may be disrupted and demethylation of H3K4me3 by Kdm5b restored with the use of Parp inhibitors. In both incidencies, transcriptional inhibition will ensue. Such drugs may also act to restore Dnmt1 methylation of DNA, further promoting gene silencing. **C.** Parp inhibition can disrupt protein: DNA interactions, preventing the maintenance of certain trans-activating factors (e.g., Nfat) at transcription start sites while also causing the retention of some repressor proteins (e.g. Dek). **D.** Gene activation may also be negatively regulated by preventing Parp from acting as a transcriptional coregulator and obstructing the recruitment of such proteins as the histone acetyl transferase p300.

1.14 | Parp-1 as a regulator of transcription

First, direct PARylation of histones can lead to a loosening of chromatin conformation. PARylation of bulk nucleosomes *in vitro* leads to decondensation (Faraone-Mennella, De Lucia et al. 1993) and Parp activity is required for chromatin loosening at stress induced genes in *drosophila* (Tulin and Spradling 2003). Biochemically, the negative charge conferred by the PAR group onto histones promotes their release from the DNA due to charge repulsion. Such is the case for histone H1, a heterochromatin-promoting factor whose PARylation-dependent removal from chromatin serves to promote chromatin relaxation (Poirier, de Murcia et al. 1982, Huletsky, de Murcia et al. 1989). Interestingly, Parp-1 localization is inversely related to histone H1 binding throughout the genome and higher proportions of Parp-1:H1 proteins tend to indicate active promoters (Krishnakumar, Gamble et al. 2008). Consistent with this, experiments using Parp-1 mutants and Parp inhibitors in *drosophila* revealed more pronounced heterochromatin at the Heat shock protein 70 (Hsp70) locus (Tulin and Spradling 2003). Likewise, an RNAi screen revealed Parp is necessary for nucleosome eviction from chromatin at the Hsp70 locus during the rapid gene induction response to heat shock (Petesch and Lis 2008). Recently, it has been demonstrated that lipopolysaccharide-induced Parp activity displaces nucleosomes from target genes, thereby facilitating transcription, as transactivating factors will not have to contend with the physical obstacle of dense nucleosomes at these promoters (Martinez-Zamudio and Ha 2012). In sum, the ability of Parp-1 to remodel chromatin in a manner conducive to transcriptional activation strongly suggests that Parp-1 primarily acts as a potent activator of transcription. However, in some contexts, Parp-1 may promote a repressed chromatin conformation when enzymatically inactive, and a more loose structure upon activation (Wacker, Ruhl et al. 2007). At a subset of promoters, under unstimulated conditions, Parp-1 presents itself in a corepressor complex with nucleolin, nucleophosmin, and Hsp70. These repressive factors, however, are released upon signal activation of Parp-1, thus providing a mechanism for differential effects of Parp-1 on chromatin structure (Ju and Rosenfeld 2006).

Countering the effects of PARylation on chromatin structure is poly(ADP-ribose)glycohydrolase (known as Parg). Parg catabolizes ADP-ribose polymers synthesized by Parp-1. This enzymatic activity has been demonstrated to impair Parp-mediated chromatin remodeling *in vitro* (Kim, Mauro et al. 2004). Chromatin remodeling mediated by Parp-1

potentiates transcriptional activation by the ER (Kim, Mauro et al. 2004) and Parg was shown to suppress estrogen-dependent transcription through blocking Parp activity. Further work is required to elucidate the *in vivo* action of Parg on chromatin structure.

In addition to histone H1 removal, Parp-1 configures chromatin through modification of proteins involved in remodeling and organizing chromatin structure. PARylation generally results in protein activation, but can also result in functional suppression of chromatin remodelers. For example, PARylation is inhibitory to the function of the repressive remodeling complex Iswi (Sala, La Rocca et al. 2008). Iswi is known to promote the association between H1 and DNA (Corona, Siriaco et al. 2007), thus illustrating a complementary mechanism by which PARylation results in a reduction of H1 binding to DNA.

Notably, Parp interaction with Brg-1 (SmarcA4), together with histone deacetylases (Hdacs), results in a repressive complex, inactivating the transcription of genes involved in cardiomyocyte differentiation through deactylation of histones (Hang, Yang et al. 2010). Thus, in particular contexts, Parp-1 activity can relay transcriptionally repressive signals. Conversely, this same study showed that a Parp-1/Brg-1 complex devoid of Hdac could activate a separate subset of genes. It remains to be seen if the PARylation of Brg1 leads to gene activation in other tissue types.

In addition, other chromatin remodelers are modified by Parp-1 under conditions of cellular stress (Gagne, Isabelle et al. 2008, Gagne, Pic et al. 2012). Proteomic studies have shown these to include TopoIIα, Brg1, TopoIIβ, HmgA1, Chd1, Chd5 and Snf2L1. Clearly, Parp activation relays a signal that is having a profound effect on chromatin structure. However, the precise impact PARylation has on these proteins, and the subsequent effects on transcription of their target genes remains unknown.

Second, Parp activity regulates gene expression through control of epigenetic mechanisms including histone modification and DNA methylation. Parp covalently modifies the epigenetic regulatory protein CCCTC binding factor (Ctcf) (Yu, Ginjala et al. 2004). Ctcf PARylation is important for its insulator activity, which functions to prevent enhancers and repressors from acting on distal promoters over long distances. Therefore, inhibition of Ctcf PARylation will result in altered regulation of target genes through the aberrant actions of distal regulatory regions.

Chromatin immunoprecipitation experiments and knockdown studies indicate Ctcf plays an import role in the maintenance of chromatin boundaries (Cuddapah, Jothi et al. 2009, Witcher and Emerson 2009). Repressive heterochromatin is the default state and, unless constrained, will spread passively throughout a chromosome (Talbert and Henikoff 2006). Chromatin boundaries form a barrier prohibiting the spread of repressive chromatin. Interestingly, Ctcf PARylation has been linked to the maintenance of chromatin boundaries at tumor suppressor genes (Witcher and Emerson 2009, Farrar, Rai et al. 2010).

This is supported by unpublished data from our lab showing Parp inhibitors lead to an accumulation of repressive histone modifications, such as H3K27me^3, at tumor suppressor genes. We have also published that Parp-1 inhibition through knockdown, or pharmacologic approaches, results in the transcriptional repression of the *Rassf1a* and *p16* tumor suppressor genes (Witcher and Emerson 2009). Based on this data, it is not unexpected that Parp inhibitors have been found to have transcriptionally repressive effects on tumor suppressor genes (Witcher and Emerson 2009, Nguyen, Zajac-Kaye et al. 2011). However, it remains to be proven that these negative effects are mediated by disruption of Ctcf function.

Beyond modulating its role as a chromatin boundary protein, PARylation of Ctcf may act as a docking site for Dnmt1 binding (Zampieri, Passananti et al. 2009, Zampieri, Guastafierro et al. 2012). This interaction is thought to act a molecular sponge, prohibiting Dnmt1 from methylating regions surrounding Ctcf binding sites. Elegant studies utilizing engineered mutations of endogenous Ctcf sites clearly show localized accumulation of DNA methylation when Ctcf binding is abolished (Pant, Kurukuti et al. 2004, Davalos-Salas, Furlan-Magaril et al. 2011). However, it remains to be proven that the interaction between Ctcf and Dnmt1 is pivotal for the capacity of Ctcf to prevent DNA methylation. Nevertheless, this model again suggests that a loss of Ctcf PARylation brought about by Parp inhibitors would result in profound epigenetic changes and significant changes to gene expression throughout the genome. Supporting this model is at least one study showing that Parp inhibition does indeed result in widespread accumulation of DNA methylation (Reale, Matteis et al. 2005).

In addition to epigenetic regulation through Ctcf, PARylation of other target proteins organize chromatin structure through coordinating the placement of histone modifications. This has been most clearly demonstrated for the histone demethylase Kdm5b (Krishnakumar and Kraus 2010). Modification of Kdm5b by Parp-1 allows H3K4me3 to persist in the promoter

regions of actively transcribed genes. H3K4me3 is important for loading the PolII machinery at most, if not all, actively transcribed genes. PARylation of Kdm5b prevents active demethylation of this mark at Kdm5b target genes, thus promoting transcription. Knockdown of Parp-1 potently blocks transcription of Kdm5b target genes through this mechanism. Clearly, small molecule inhibitors or Parp-1 would be expected to disrupt transcription in a similar fashion.

Third, Parp activity controls the recruitment and maintenance of transcription factors to promoter regions. As stated above, classic experiments from the Roder lab demonstrate that Parp copurifies biochemically with RNA PolII (Slattery, Dignam et al. 1983). Follow up work showed Parp enhances the assembly of the preinitiation complex *in vitro* (Meisterernst, Stelzer et al. 1997). Surprisingly, Parp activity has been demonstrated to be necessary to retain Pol II at actively transcribed target genes (Zobeck, Buckley et al. 2010). It is postulated that PAR polymers creates a scaffold that retains Pol II at gene loci. It is quite possible, but remains to be proven, that PAR scaffolds act to retain transactivators at target genes as well. The trans-activating factors Nfat, Klf8 and Tef-1 are associated with, and activated by PAR polymers (Butler and Ordahl 1999, Olabisi, Soto-Nieves et al. 2008, Lu, Wang et al. 2011). It is possible the mechanism lies in the retention of these factors at target genes by (ADP)ribose polymers.

In contrast to this model, PARylation of the transcriptional repressor Dek by Parp-1 serves to evict Dek from chromatin, ultimately promoting gene activation (Gamble and Fisher 2007). Thus, blocking the actions of repressors serves as another mechanism through which Parp activates transcription.

Fourth, Parp-1 itself has also been described to act as a coregulator of transcription. It is recruited to genes via interaction with DNA binding factors. As a coregulator, Parp-1 can be integrated into complexes having stimulatory effects on transcription mediated by transcription factors such as NF-κβ and AP-2 (Li, Naidu et al. 2004). To complete the assembly of the NF-κβ activating complex, Parp-1 is required for the integration of the histone acetyltranferase p300(Hassa, Buerki et al. 2003, Kaur, Chen et al. 2006), providing another link between Parp -1 and histone modification. Parp-1 is also a component of a coactivating complex responsible for driving nuclear hormone receptor-mediated transcription in response to estrogens and retinoids (Pavri, Lewis et al. 2005, Ju, Lunyak et al. 2006).

Docking with Parp-1 can also serve to enhanced phosphorylation of the associated transcription factor leading to heightened trans-activating capabilities. This has been

demonstrated for B-Myb in a cell cycle dependent fashion (Cervellera and Sala 2000, Santilli, Cervellera et al. 2001) and Elk-1 in response to Erk-1 activation (Cohen-Armon, Visochek et al. 2007). These data indicate Parp activity links intra and extracellular signaling events with gene induction.

Parp-1 knockdown studies show Parp activity also functions to repress a subset of genes (Frizzell, Gamble et al. 2009). Consistent with this, PARylation of a number of transcription factors has been described to prevent their interaction with DNA. This has been described for Smads (after TGFβ stimulation) (Lonn, van der Heide et al. 2010), p53 and Sp1 (Kumari, Mendoza-Alvarez et al. 1998, Malanga, Pleschke et al. 1998, Zaniolo, Desnoyers et al. 2007). Inactivation of Smads by Parp following TGFβ signaling remains controversial as a more recent study found Parp necessary for Smad activation post-TGFβ exposure (Huang, Wang et al. 2011). It will be of great interest in the future to determine how Parp integrates signaling events such as TGFβ, reactive oxygen species and growth factors into coordinated transcriptional outputs.

Information from *in vitro* studies describing the inhibition of transcription factor binding to cognate sites by PARylation should be taken with caution. Without the constraints found *in vivo*, ADP-ribose polymers can be extended to enormous lengths *in vitro* (D'Amours, Desnoyers et al. 1999, Mendoza-Alvarez, Chavez-Bueno et al. 2000). It is likely that the formation of such a network of polymers could impair binding to cognate DNA sites *in vitro* due to simple steric hindrance.

Overall, evidence suggests that Parp-1 plays primarily a stimulatory role on transcription, including activation of tumor suppressor genes and a more minor role in gene repression. Clearly there is overwhelming data demonstrating that Parp-1 participates in gene regulation at multiple levels, most prominently by coordinating transcription factor activity and organizing chromatin structure. It is imperative that these aspects of Parp-1 function be considered, along with its role in DNA damage repair if we are to extend the clinical use of Parp inhibitors to treat tumors beyond those bearing Brca mutations.

1.15 | Clinical modulation of the Poly(ADP)ribose pathway: future perspectives

The epigenetic, chromatin remodeling, and transcriptional regulatory roles of Parp-1 are necessary to activate a group of genes under basal conditions and another cohort in response to

stimuli, such as cell stress. Therefore, inhibiting Parp-1 will potentially disrupt expression of a wide range of genes, including tumor suppressor genes, which may limit the benefits of Parp inhibitors in Brca wild-type patients.

That being said, can this pathway be targeted successfully to treat a broad range of tumors? In our opinion, yes, but we need to revisit our approach. First, it is clear that Parp-2 can compensate for Parp-1. Thus Parp inhibitors should be tested for their capacity to block the activity of both proteins before being considered for clinical trials. Such consideration might have prevented the failed clinical trials with iniparib, a drug initially described as a Parp inhibitor, but that has recently been proven to lack such activity (Patel, De Lorenzo et al. 2012).

Second, the concept of Brcaness needs to be more clearly defined using both genetic and epigenetic markers. It is probable that tumors with epigenetically silenced, as well as mutated, DNA repair genes will be sensitive to Parp inhibitors. This concept may also be employed to predict successful combinations of chemotherapeutics with Parp inhibitiors. Further, it is possible that epigenetic silencing of tumor suppressor genes that are Parp targets may negate any pro-proliferative effects of Parp, potentially rendering the cells sensitive to Parp inhibition. To date, *in vitro* models have been used to accurately predict tumor pathologies that are clinically sensitive to Parp inhibitors (Bryant, Schultz et al. 2005, Donawho, Luo et al. 2007). Therefore, future testing of Brcaness models using *in vitro* systems will be an important stepping stone to make these models clinically relevant.

1.16 | The potential of Parg inhibitors for therapy

Finally, we propose that Parg represents an attractive therapeutic target (Figure IV). The understudied protein Parg counteracts the activity of Parp by removing PARylation marks from target proteins. Unlike Parp that has numerous encoding genes throughout the genome, only one Parg-encoding gene exists in the cell. However, several isoforms of Parg are detected as a result of differential splicing and the use of alternative transcription start sites from the single gene locus described (Min and Wang 2009). Of the different Parg variants, only the 111kDa form is nuclear, and ultimately has implications in DNA stability and regulation. Parg knockout mice exhibit an embryonic lethal phenotype, a testament to the importance of this gene as well as its lack of redundancy in the cell (Koh, Lawler et al. 2004).

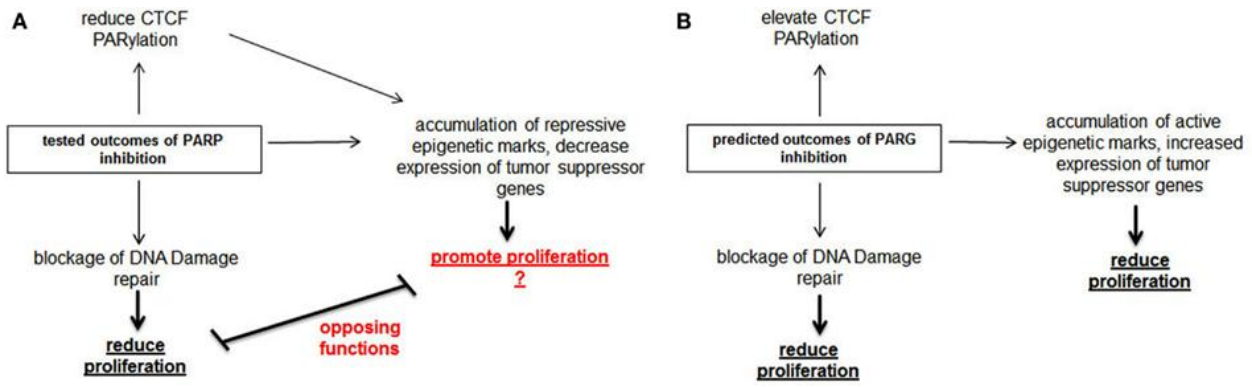

Figure IV. Comparison of molecular action of Parp inhibitors with predicted action of Parg inhibitors. **A.** Parp inhibitors inhibit the growth of Brca mutated tumors through blocking DNA damage repair. Parp inhibitors also dePARylate Ctcf, cause the accumulation of repressive epigenetic marks at tumor suppressor genes and diminish the expression of these genes. We propose that these properties of Parp inhibitors will limit their usefulness as anti-cancer therapeutics. **B.** In contrast, we predict Parg inhibitors will stimulate Ctcf PARylation and the transcription of tumor suppressor genes, in addition to blocking the DNA damage repair response. Thus, Parg inhibition may have greater potential as an anti-cancer therapeutic than Parp inhibitors.

Intriguingly, Parg has a critical role in DNA damage repair, similar to Parp-1 (Fisher, Hochegger et al. 2007, Mortusewicz, Fouquerel et al. 2011). Counterintuitively, while PARylation of target proteins is necessary to initiate the repair response to DNA damage, removal of these tags is essential for a complete DNA damage response (Min, Cortes et al. 2010). This may be due to the fact that auto-PARylation of Parp inhibits the enzyme's function and promotes its release from DNA (D'Amours, Desnoyers et al. 1999). Parg activity may be required to remove the inhibition of Parp and restore its DNA binding. Ultimately, this cyclic addition and removal of PAR appears to be required for effective DNA damage repair. This correlates with findings of an impaired DNA damage response in Parg 110KO MEFs, with reports of increased Rad51 foci (Min, Cortes et al. 2010). This defect may explain the increased sensitivity of Parg knockout cells to chemotherapy. Likewise, Parg deficient trophoblast stem cells are also more sensitive to treatment with UV. In response to DNA damage, these cells permit the release of the apoptosis inducing factor to mediate cell death (Zhou, Feng et al. 2011). This serves as another pathway through which Parg inhibition can act to promote cell death in

cancer. Furthermore, treatment with gamma rays or alkylating agents also result in an increased rate of sister chromatid exchanges and overall increases in chromosomal instability in Parg knockout cells (Cortes, Tong et al. 2004). These defects recapitulate much of those found in Parp knockout cells, suggesting that both the addition and removal of PAR is responsible for genome stability. Thus, it seems that testing Parg inhibitors in combination with chemotherapy or radiation therapy may work synergistically to inhibit cell growth, ultimately providing some clinical relief from the disease. Given the role of Parg in DNA damage repair, it can be predicted that Parg inhibition would likely create a synthetic lethal situation in Brca defective cells in a similar manner as Parp inhibitors. Consistent with this concept, Brca mutated cells have been shown to be highly sensitive to an inhibitor of Parg (Fathers, Drayton et al. 2012). Further, we now have exciting new data demonstrating Parg inhibition is a relevant approach to inhibit the proliferation of triple-negative breast cancer cell both *in vitro* and *in vivo* (manuscript under review).

Considering the role of Parp and Parg in gene expression, it is noted that Parp is an important activator of transcription and probably plays an important role in promoting transcription of tumor suppress genes while Parg may block Parp-1 mediated chromatin remodeling and transcriptional activation in specific circumstances. Therefore, we postulate Parp inhibitors may actually have pro-oncogenic effects on some cell populations, but Parg inhibitors might heighten the effects of PARylation, thus promoting the transcription of tumor suppressor genes. Further, in cancer cells having defects in the PARylation pathway such as aberrantly dePARylated Ctcf, Parg inhibition might serve to correct these deficiencies.

Supporting these rationale for Parg inhibition being a novel approach for anti-cancer therapy are several reports indicating Parg inhibition has potent anti-tumor effects against cholangiocarinoma *in vivo* (Marienfeld, Tadlock et al. 2003), and *in vitro* data showing growth inhibitory activity of Parg inhibition or knockdown in multiple types of cancer (Fauzee, Li et al. 2012, Feng, Zhou et al. 2012, Li, Li et al. 2012, Pan, Fauzee et al. 2012). Now that the crystal structure of Parg has been solved (Slade, Dunstan et al. 2011, Kim, Kiefer et al. 2012), there is a clear need to develop specific inhibitors of this enzyme and test their efficacy as anti-cancer therapeutics.

2. PURPOSE

While the potential of Parg inhibitors seems promising, the main questions arising are whether Parg acts as a driver of cancer and whether it can be targeted for therapy in breast cancer. Answering these questions was the main focus of this Masters project with the primary goal being to characterize the extent of Parg involvement in oncogenesis by studying the effect of Parg and PARylation in cellular transformation and expression of Ctcf target genes. The secondary goal was to explore Parg inhibition as a potential therapeutic approach in breast cancer.

3. HYPOTHESIS

Our model suggests that Parg may become upregulated in certain cancers and result in the dePARylation of Ctcf, disrupting its ability to maintain chromatin boundaries. Consequently, repressive heterochromatin could infringe over TSG loci and mediate gene silencing. The loss of growth control regulators in the cell as a result of heightened Parg levels can contribute to cancer initiation and progression (Figure V).

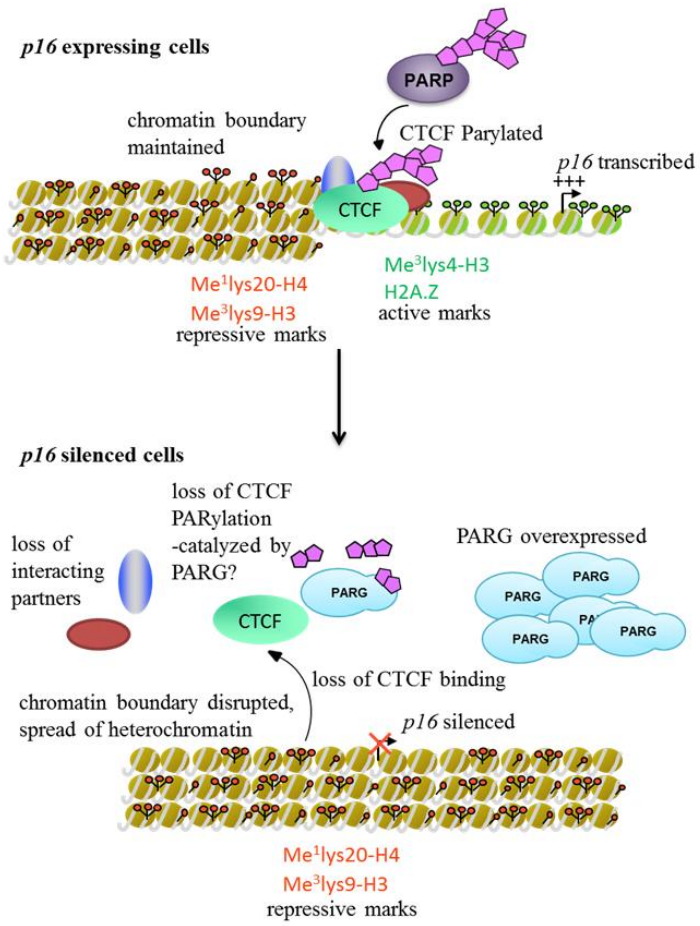

Figure V. Proposed model of Parg-induced tumor suppressor gene silencing. In untransformed cells, Ctcf is PARylated and bound to its target sites throughout the genome. This binding establishes a chromatin boundary preventing the infringement of heterochromatin onto tumor suppressor gene loci and allowing the expression of these factors to be maintained. In certain cancers, Parg may become upregulated, causing the loss of Ctcf PARylation. In turn, this promotes the loss of the chromatin boundary and spread of heterochromatin, ultimately contributing to tumor suppressor gene silencing and oncogenesis.

4. AIMS

The first step in characterizing Parg as a driver of cancer was to determine the extent of clinical cases where Parg overexpression is detected. For this, a variety of bioinformatics tools were used to looking at the mRNA expression of Parg in cell lines and clinical samples. This data was supplemented with western blot data of cell lines grown in culture as well as immunohistochemistry data from tissue arrays of breast cancer samples.

Following the description of Parg overexpression in patient tumors, it was important to test the effect of decreased Parg levels in the cell as well as the potential of Parg inhibitors in slowing cell growth. Using Parg knockouts and the Parg inhibitor tannic acid were critical for this purpose.

Alternatively, we hoped to create stable primary breast cell lines overexpressing Parg to characterize this protein as a driver of cancer. Doing so entailed testing the overexpressing cell lines by a variety of growth and transformation assays and looking for changes in gene expression.

We predict CTCF may be an important target of PARG and that CTCF dePARylation can mediate the pro-oncogenic effects of PARG. Therefore, we will test the effect of Ctcf mutants, deficient in PARylation, on cell growth and gene expression. This would help elucidate the role of Ctcf PARylation in regulating TSGs and preventing pro-oncogenic molecular events.

5. MATERIALS & METHODS

5.1| Cell culture reagents

Mcf10a cells were grown in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) supplemented with 10 µg/mL insulin, 0.5 µg/mL hydrocortizone, 20 µg/mL epidermal growth factor, 0.1 µg/mL cholera toxin and 2% horse serum, hMLE and HMECs were kept in Mammary Epithelial Basal Medium (MEBM) supplemented with EGF, insulin, hydrocortisone and bovine pituitary extract from Lonza, Hek293T, T47D, BT-474 cells were grown in DMEM 10% fetal bovine serum and MDA-MB-468, BT-20, MDA-MB-435 were grown in Roswell Park Memorial Institute medium (RPMI)1640 with 10% FBS cultured at 37^{o}C and 5% CO_2. MDA-MB-175VII, on the other hand, were cultured in Leibovitz's L15 medium 10% FBS and incubated at 37^{o}C with ambient CO_2 levels, as recommended by ATCC. 67NR, 168FARN, 4T07, 66c14 and 4T1 cells were grown in DMEM supplemented with 15% FBS, Penicillin/Streptomycin and 5mM nonessential amino acids. These were then injected into mice, allowed to form tumors and were subsequently excised and kept for further analysis.

Tannic acid was purchased from Sigma and was prepared fresh for each drug treatment in PBS. Tannic acid was repurchased regularly to maintain stocks that were not oxidized through exposure to air. Doxorubicin was purchased from Sigma, dissolved in water and frozen stocks were stored at -20^{o}C until use.

5.2 | Generation of Ctcf mutant constructs

Mutational cloning was carried out according to the protocol described by the Gruenert group and detailed here (Xu, Colosimo et al. 2003). A 24 base-pair reverse primer containing the desired mutation within the Ctcf target gene (refered to as the mutagenic primer) in addition to two external primers (forward and reverse) flanking the sequence were designed. For the first round of PCR reactions, a mix of 1ng template DNA, 10pmol of the forward external primer, 10pmol of the mutagenic primer, 0.25mM dNTPs, *Pfu* polymerase and 1x polymerase reaction buffer was prepared. A PCR amplification was then carried out as follows: 94^{o}C for 3 min; 25 cycles of 94^{o}C for 20 sec, 58^{o}C for 20 sec and 72^{o}C for 1-2 min; 72^{o}C for 5 min. Following a

1:25 and a 1:50 dilution of the PCR product derived, 2uL was added to a PCR mix containing all the components described above using reverse external primer instead of the mutagenic primer. With the same PCR conditions, a second amplification reaction was run. The PCR product was isolated using the PCR extraction kit by Promega according to the manufacturer's protocol and sent for sequencing to verify that the fragment generated was as desired. These fragments were then PCR amplified with EcoRI cut sites for cloning into the pBABE retroviral vector. The Ctcf fragments containing the EcoRI cut sites in addition to the pBABE retroviral vector were digested with EcoR1 (Fermentas) overnight at 37°C. The following day, the digested products were run in a 0.8% agarose gel. Fragments os the correct molecular weight were isolated from the gel and purified with QIAgen quick spin columns, as per manufacturer's instructions. The Empty vector was treated with FastAP Thermosentive Alkaline Phosphatase (Fermentas) and incubated at 37°C for 65 min at 75°C to prevent self-ligation of the plasmid. Ligation of insert and linearized vector was carried out using T4 DNA Ligase (Fermentas) overnight at room temperature.

5.3| Transfection

Cells were seeded to be 60-80% confluent on the day of transfection. All transfections were carried out using polyethylenimine (PEI) in Opti-MEM with a 1:5 ratio of plasmid to PEI. The transfection mix was vortexed thoroughly, incubated for 15 min before addition to cells and was replaced with fresh complete media after 5 hours. Cells were incubated 48 hours before collection.

5.4| Retroviral generation of stable Parg-overexpressing cell lines

Hek293t phoenix cell lines described by the Nolan and Pelicci group (Grignani, Kinsella et al. 1998) were transfected according to the directions specified above with the PARG transgene packaged in the pBABE retroviral vector or the empty vector alone (using Lipofectamine 2000 instead of PEI as the transfection reagent). These cells are modified Hek293T cells that express the retroviral gag-pol and envelope genes required for the production of viruses under the control of non-moloney promoters. This cell line is easily transfectable and permits the production of virus for an extended period of time due to their enhanced ability to retain transiently transfected episomes. The morning after transfection, the media was replaced and cells were incubated for

48 hours. The virus infected media was subsequently added to the untransformed Mcf10a cell lines with hexadimethrinebromide (4µg/mL) and incubated at 37^{o}C for 72 hours. Cells were then selected with 1ug/mL of puromycin for 72 hours. Parg overexpression was confirmed by Western blotting (described below) using a monoclonal anti-Parg antibody (EMD Millipore). Parg knockdowns were generated in a similar manner using lentiviral shRNAs specific to Parg or scrambled control purchased from Sigma (Mission shRNA).

5.5| Trypan blue exclusion assay

Growth curves were generated by plating 10^5 cells in 6-well dishes. Cells were treated with 10 µM tannic acid, replenished daily. For the determination of cell number, cells resuspended in media were diluted in an equal volume of trypan blue, followed by manual cell counting using a hemocytometer on days 1, 3 and 5 of the experiment.

5.6| Western blotting, immunostaining and antibodies

Cells were disrupted with lysis buffer (20mM Tris pH 7.5, 420mM NaCl, 2mM $MgCl_2$, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.5% Triton, 1x P8340 (Sigma), 1mM PMSF, 1mM DTT, 2mM NaF, 10mM BGP) for 35 min on ice followed by a 25 min spin at maximum velocity to pellet debris. The supernatant was then removed and quantified using the Bradford reagent. The OD_{595} of each sample was then measured using a spectrophotometer and compared to a standard curve prepared with bovine serum albumin. An equal concentration of sample was then separated using standard Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis (SDS-PAGE) techniques. Proteins were then transferred overnight at 35V and 1hour at 70V to BioRad nitrocellulose membranes. These were blocked with 5% skim milk in 1x Tris Buffered Saline and 0.1% Tween (TBS-T) for 1 hour at room temperature and probed overnight at 4^{o}C with the antibodies listed below at a dilution of 1:1000. The day after, membranes were washed thrice in TBS-T and were incubated with secondary anti-mouse or anti-rabbit horseradish-peroxidase for 1hour at room temperature. This was followed by three additional washes in TBS-T. Enhanced ChemiLuminescnece (ECL) substrate was added to the membranes and these were exposed to capture the differences in protein levels in the cells. The antibodies used as probes for Western and IHC were as follows; poly (ADP-ribose), poly (ADP-ribose) glycohydrolase, phospho-

H2A.X, H3K27me3, Ctcf (EMD Millipore), Ctcf, poly (ADP-ribose) polymerase -1/2 , Brg-1, laminB, β-tubulin, C23/nucleolin (Santa Cruz), β-Actin (Sigma), Ctcf, HA-tag (Abcam) and Ctcf (BD Biosciences).

5.7| Immunohistochemistry

Immunohistochemistry was carried out by the Molecular Pathology core facility at the Jewish General Hospital using standardized procedures with the Ventana Discovery automated immunohistochemistry machine. Tissue arrays were purchased from US Biomax.

5.8| Immunofluorescence

Cells were grown on sterile coverslips and treated appropriately until ready for analysis. On the day of the experiment, cells were rinsed in PBS and fixed in freshly prepared 4% paraformaldehyde for 15 min at room temperature. Following three 10-min washes in PBS, cells were permeabilized with a 3% BSA solution prepared in PBS with 0.2% Triton X-100 for 5 min. Again, slides were washed thrice for 10 min in 3% BSA in PBS. Cells were then incubated with a 1:200 dilution of anti-phospho H2A.X (Millipore) for 1 hour at room temperature. Following three more washes in 3% BSA in PBS, cells were incubated with secondary antibody conjugated to the fluorescent probes Alexa 488 at a dilution of 1:1000 in PBS/BSA for 1 hour at room temperature in the dark. The slides were extensively washed in PBS (4x 10min) and then incubated in 300nM DAPI for 5 min. These were then rinsed in PBS three times. Slides were mounted on coverslips using the ProLong Gold Antifade Reagent from Invitrogen and images were captured using the EVOS fluorescent microscope.

5.9| Immunoprecipitation (IP)

Cells were lysed for 35min in lysis buffer (20mM Tris pH 7.5, 420mM NaCl, 2mM $MgCl_2$, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.5% Triton, 1x P8340 (Sigma), 1mM PMSF, 1mM DTT, 2mM NaF, 10mM BGP), spun for 25min at max speed to pellet debris and the cell lysate was collected and quantified using the Bradford Reagent, as described above. A total of 1-2mg of protein was used for the IP depending on the amount of protein extracted. This was subsequently diluted 5x or more in IP buffer (20mM Tris pH 7.5, 50mM NaCl, 10mM $MgCl_2$,

2mM EDTA, 0.1% Triton X-100, 1x P8340 (Sigma), 1mM PMSF, 2mM NaF, 10mM BGP). 50uL of 50% freshly prepared protein G sepharose beads (GE Healthcare Life Sciences) were used per tube to preclear the sample. Following a 2 hour incubation at 4^{o}C on a nutator, beads were pelleted by a 3 min spin at 5000rpm. The lysate was then collected and 2-3ug of antibody were added to each sample and these were kept on a nutator at 4^{o}C overnight. The following day, samples were incubated for 2-4 hours with 25uL of 50% beads. Following 4 washes in IP buffer, beads were heated at 100^{o}C in SDS loading buffer to release the proteins from the beads. This was loaded onto an 8% polyacrilamide gel and treated according the directions described in the Western blot protocol.

6. RESULTS

6.1| PARG is overexpressed in certain cancers

Employing a variety of online databases as resources, we observed that Parg mRNA is overexpressed in a significant proportion of breast cancer cell lines and tumor samples. Specifically, approximately one third of 683 breast cancer cell lines analyzed from the *Yau* expression profile from the UCSC Cancer Genome Browser tested high for Parg mRNA (Figure 1A) (Yau, Esserman et al. 2010). Likewise, data from the Oncomine database revealed that approximately half of 122 ductal breast carcinomas have elevated Parg transcripts (Figure 1B). This is not the case for all cancers, including glioblastoma multiforme where Parg is underexpressed in almost all cases (Figure 1A). If we consider the breast cancer subtype of the cell lines positive and negative for Parg mRNA expression, we see an enrichment of the luminal subtype in Parg overexpressing cell lines and an enrichment of the basal subtype in cell lines with low levels of Parg mRNA (Figure 2). Thus, online bioinformatics resources have revealed that Parg is overexpressed at the mRNA level in cell lines and tumor tissue, and that this trend is preferentially observed in luminal cells.

Similar to the findings observed at the mRNA level, higher levels of Parg protein are detectable in a significant number of breast cancer samples analyzed and this is correlated to increased malignancy and poor clinical outcomes. Analysis by Western blot revealed that such breast cancer cell lines as T47D, BT-20 and MDA-MB-231 are significantly enriched in Parg protein relative to the untransformed control hMLE, HMEC and hTERT cells (Figure 3A). Interestingly, while at the mRNA level, it seemed as though the basal cell types were Parg deficient, these findings did not stand at the protein level. In fact, the basal BT-20 and MDA-MB-231 cells, while having been described to possess low Parg mRNA, have been described here to have high Parg protein. Even the triple-negative MDA-MB-435 and MDA-MB-468 cells were found to have slightly higher Parg levels than the untransformed cell lines. In addition to cell line data, we have immunohistochemistry data showing that several tumor samples express high levels of Parg in comparison to normal tissue (Figure 3B). The localization of Parg varied, at times residing in the nuclear fraction, and at others residing in the cytoplasmic fraction.

Assessing the status of Parg expression over the course of cancer progression as well as in relapse-free survival outcomes for patients was also important for determining the extent of Parg involvement in cancer. Indeed, there appears to be a trend towards Parg overexpression and advanced stages of breast cancer. To link Parg levels with different stages of tumor evolution, we used the 4T1 mouse model of breast cancer progression established by F Miller (Miller, Miller et al. 1983). This model revealed that Parg protein levels are elevated in more aggressive mouse tumors (Figure 4A). For instance, the metastatic 4T07 and 66c14 cells are significantly more enriched for Parg protein than the spontaneously arising 67NR cells. This is complemented by data showing that Parg overexpression was correlated to poor clinical outcomes in luminal B, node negative breast cancer (Figure 4B). Interestingly, a similar outcome was detected for stage 1 and 2 ovarian cancer patients, extending the range of cancers in which Parg may play a role. Thus, our analysis reveals enrichment of Parg protein in breast cancer cells, particularly in advanced stages of the disease and we correlate this to worse clinical outcomes.

To begin characterizing the role of Parg in cancer, we searched online databases for correlations between *PARG* expression and that of other genes. Interestingly, data from the Neve database from the UCSC cancer browser (Neve, Chin et al. 2006) revealed an inversely proportional correlation between *PARG* mRNA and expression of the tumor suppressor genes $p16^{INK4a}$, $p15^{INK4b}$ and *RASSF1a* in a significant number of cell lines examined (Figure 1C). Specifically, among 18 cell lines with high *PARG* levels, 60% underexpressed $p16^{INK4a}$, 55% underexpressed $p15^{INK4b}$ and 50% had low levels of Rassf1a. Also of interest was the *ZMYND10* gene, closely positioned to the *RASSF1a* gene at a locus that is often the target of epigenetic silencing or allelic loss (Riquelme, Baez et al. 2004, Tischoff, Markwarth et al. 2005). Results from the UCSC browser screening several breast cancer cell lines demonstrate a strong correlation between *RASSF1a* and *ZMYND10* expression and this is inversely correlated to *PARG* expression. *PARG* is not correlated with the expression of all TSGs, however, as is illustrated for the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (*MGMT*) and the detoxification enzyme Glutathione S-transferase P1 (*GSTP1*). Interestingly, the levels of $p16^{INK4a}$, $p15^{INK4b}$ and *RASSF1a* were not correlated to another epigenetic regulator *EZH2*, suggesting that Parg may be a specific and important regulator of the expression of these key genes. This evidence reveals a potentially strong selection for Parg overexpressing cells to either delete or epigenetically silence these important TSG loci. In agreement with these results, the

cell lines found to have high Parg protein are also those having been reported in the literature to have epigenetically silenced *RASSF1a* (Singal, Vanwert et al. 2005, da Costa Prando, Cavalli et al. 2011). The $p16^{INK4a}$ gene is often deleted in cancer, particularly in cell lines that need to be maintained in a proliferative state to be sustained in tissue culture. However, among the cell lines that retain the $p16^{INK4a}$ gene, those with high Parg protein expression have been found to have a methylated promoter at this site, while cells with low or intermediate Parg levels are unmethylated, according to the literature (Bisogna, Calvano et al. 2001, Silva, Silva et al. 2003). Other factors such as the expression of *ER* and *HER2* do not seem to correlate well with Parg overexpression, at least at the mRNA level. This alludes to the possibility that Parg overexpression may be a relevant factor in some triple-negative breast cancers.

Overall, the strong correlation between Parg expression and oncogenicity found at the RNA and protein level suggests that it is worth studying this protein to assess its possible involvement in driving cancer and its potential to be targeted for breast cancer therapy. Our investigation thus far has opened the door towards the possibility of epigenetic silencing of TSGs as a possible outcome of Parg overexpression in cancer. While clinically, there appears to be the strongest link between Parg and luminal B cancers, our data and analysis suggest that Parg may also play a role in triple-negative cases.

6.2| Biochemical overexpression of Parg in untransformed cell lines

Having established elevated Parg levels in breast cancer, we went on to characterize whether this protein has any impact on inducing cellular transformation. For this, the Parg transgene was inserted into the pBABE-retroviral vector (Morgenstern and Land 1990) with a FLAG-tag for use in overexpression experiments. A transient transfection of Hek293t phoenix cells with this vector effectively increased Parg expression in these cells and likewise efficiently reduced cellular PARylation levels (Figure 5). This experiment revealed that the vector of interest was indeed catalytically active and appropriate for use in our study. While this transient transfection model recreated a state of PARylation depletion that was attractive for study, this is not an ideal model for studying transformation as Hek293t are already tumorigenic. Instead, we decided that overexpressing Parg in untransformed cell lines such as Mcf10a would be a more interesting and relevant model to study. Any effect of Parg on these cells would be a more

accurate representation of a breast cancer model than using a cell line that has already been transformed. These cells, however, are difficult to transfect, leading us to use retroviral infections to obtain the overexpression desired. This method was successful and it was possible to generate Parg-overexpressing Mcf10a cells (Figure 6A). This change was followed by a noticeable difference in morphology in these cells (Figure 6B). In fact, the changes suggest an EMT transition induced by Parg overexpression. Interestingly, these changes were met with a decrease in proliferation abilities of the Parg-overexpressing cells in culture (Figure 6C). Unfortunately, after two weeks in culture, all available stocks of these stables were found to have silenced the transgene and it was not possible to perform further analyses on these. Although the reason for this loss of expression is unknown, we speculate that this may be due to silencing of the pBABE plasmid because of intrinsic properties of the vector or that PARG overexpression was selected against. We are currently in the process of generating new Parg-overexpressing Mcf10a cells with which we will rapidly coordinate experiments before the transgene expression is lost.

6.3| Tannic acid inhibition of Parg slows the growth of breast cancer cell lines

While generating the overexpressing stable cell lines, we went on to study the effect of inhibiting Parg in various cell lines to characterize the relevance of this protein as a target for therapy. For this purpose, we used tannic acid, a cell permeable Parg inhibitor (Figure 7A) (Tanuma, Sakagami et al. 1989, Tsai, Aoki et al. 1992, Rapizzi, Fossati et al. 2004, Fathers, Drayton et al. 2012).

As a proof-of-principle, one luminal and one basal cell line (T47D and MDA-MB-468 respectively) were treated with 10uM tannic acid at several time points throughout one day and the PARylation status of cells was assessed. Consistent with a role for tannic acid in Parg inhibition, treatment with the drug increased global levels of PARylation in both cell lines (Figure 7B). At 24 hours, PARylation levels decreased back to normal conditions, likely the result of drug metabolism. In fact, at 24h, PAR levels were lower than in untreated cells, likely an overcompensation before PAR levels stabilize.

Looking at the effect of the drug on cell proliferation, it was clear from previous work in the lab that tannic acid has an inhibitory effect on growth. The triple-negative cell lines MDA-MB-468 and MDA-MB-435 were very sensitive to tannic acid, with a more subtle effect on the luminal BT-474 cells (Figure 8). Parg knockdowns in T47D and MDA-MB-468 cells reduced cell growth to a similar extent as tannic acid treatment (Figure 9). Moreover, using tannic acid on the knockdown cells did not provide any further reduction in cell growth. Together, the data suggest that tannic acid works through Parg to reduce the rate of cellular proliferation. This reveals an exciting potential for Parg inhibition to treat breast cancer. This potential can likely also be extended to triple-negative cancers given the impressive effect tannic acid has on the growth rate of MDA-MB-468 and MDA-MB-435 cells. The effect observed on these cells is remarkable given that no other drugs function on triple-negative cells as efficiently (Foulkes, Smith et al. 2010).

Exploring the mechanism by which tannic acid inhibition of Parg can mediate this effect, we decided to explore the impact of DNA damage on cell growth. Parg is known to be involved in the DNA damage repair process (Min, Cortes et al. 2010) and thus, it was important to characterize the extent of genetic lesions occurring in the cell in response to tannic acid. This was particularly important given that DNA damage is known to stall cell cycle progression until sufficiently repaired and may serve as a mechanistic explanation of the effect of tannic acid on cell growth (Noguchi 2010). Thus, we probed samples treated with tannic acid for phospho-H2A.X Ser 139 to assess the extent of DNA damage occurring in the cell. Over the course of 24h, there was no significant effect of tannic acid treatment on DNA damage in T47D cells, assayed by Western blot (Figure 10A), and this was confirmed by immunofluorescence data for MDA-MB-468 cells (Figure 10B). Hence, the effect of DNA damage on increasing PARylation levels through Parp overactivity can be excluded, suggesting that this effect is mainly due to a deficiency in the dePARylation process. Over a longer time course, however, increased DNA damage is detected by immunflurorescence, suggesting that DNA damage plays a role in reducing the rate of cellular proliferation, over an extended period of time.

In addition to considering DNA damage as a mechanism of growth control in the cell, we hoped to assess whether Parg inhibition can promote an epigenetic effect in the cell which may serve a similar purpose. In the literature, Ctcf has been described to be important for maintaining

boundaries that specifically separate H3K27me3 enriched regions from an adjacent H3K27me3 depleted region (Figure 11A) (Barski, Cuddapah et al. 2007, Cuddapah, Jothi et al. 2009). As a result, we tested cell extracts for global changes in the repressive histone mark H3K27me3. In particular, we hoped to define changes in this mark that may explain the strong inverse correlation between Parg expression and that of TSGs like $p16^{INK4a}$ and *RASSF1a*. In agreement with our model, tannic acid lowered the levels of this repressive histone mark, alluding to a role for Parg inhibition in gene expression (Figure 11B). This could have important functional roles if the genes targeted are TSGs.

Taken together, these investigations into chemical inhibition of Parg strongly suggest that this target can have significant impacts on slowing breast cancer progression. These benefits appear to be increased in triple-negative breast cancers. Mechanistically, it appears as though there is a role for both DNA damage and epigenetics in mediating growth control. While the effect of tannic acid on DNA damage has been extensively characterized by our lab in an article currently under review, the epigenetic aspect still remains to be explored. Based on the literature, we speculate that the main influence of Parg on epigenetics will result from the dePARylation of Ctcf. As such, it was important to establish an efficient model to study Ctcf PARylation and lack therof.

6.4| Detecting Ctcf PARylation

Many studies in the literature use immunoprecipitation experiments to test for the modification of proteins by PARylation including Ctcf (Gagné, Isabelle et al. 2008, Witcher and Emerson 2009). However, the Klenova group has proposed that this modification can easily be detected directly by Western blot (Docquier, Kita et al. 2009). Specifically, they report two molecular weight bands, one of 130kDa and the other of 180kDa, that correspond unmodified and PARylated Ctcf respectively. This method is intriguing as it facilitates the process of detecting this post-translational modification and avoids the time consuming and costly immunoprecipitation method. For these reasons, we decided to further explore this approach and went on to assess the expression of Ctcf in various cell lines using commercially available Ctcf antibodies. Probing with these various antibodies, it was possible to detect several higher molecular weight bands, in some cases, that may correspond to PARylated Ctcf. To definitively

characterize which bands corresponded to modified Ctcf, these samples were juxtaposed to samples treated with 5mM of the Parp inhibitor 3-aminobenzamide (3-ABA). The rationale for this is that the drug treatment should cause the disappearance of the band corresponding to PARylated Ctcf. Observing the PARylation in cells that were treated in the presence or absence of 3-ABA revealed that the drug treatment worked (Figure 12A). When considering the bands obtained after probing with Ctcf antibodies, it was noted that while there were several bands that differed between the treated and untreated cells, no consistent band disappearance was observed in response to 3-ABA treatment (Figure 12B). Thus, it appears that detection of PARylated Ctcf is not possible given the currently available antibodies in our hands. As a result, we concluded that co-immunoprecipitation of the PAR modification and probing to detect Ctcf is still currently the best method for detection of the modification.

6.5| Characterizing Ctcf mutants deficient in PARylation

In parallel to Parg overexpression experiments, we hoped to consider models where Ctcf was PARylation-deficient. Based on a publication by Klenova et al., we generated CTCF mutants that are PARylation deficient (Figure 13). Specifically, we mutated several N-terminal domain glutamic acids residues to alanine to generate the mutant 1, having been previously characterized to be incapable of modification by PARylation. In addition, we generated a mutant 2 with fewer modifications, in hopes of teasing out the specific residues targeted by this post-translational modification. These mutants were HA-tagged in order to facilitate immunoprecipitation experiments.

We proceeded to conduct experiments to verify which of our two Ctcf mutants could not be PARylated in the cell. To do so, we transfected Hek293t cells with the Ctcf transgenes and went on to conduct immunoprecipitation assays to characterize the PARylation status of the mutants in comparison to the wild-type Ctcf. Specifically, we immunoprecipitated the PAR modification and probed for the HA tag (Figure 14). These experiments revealed that mutant 2, and not mutant 1, was deficient in PARylation. Thus, Ctcf mutant 2 can be used to study the effect of a deficiency of Ctcf PARylation in cells.

7. FIGURES

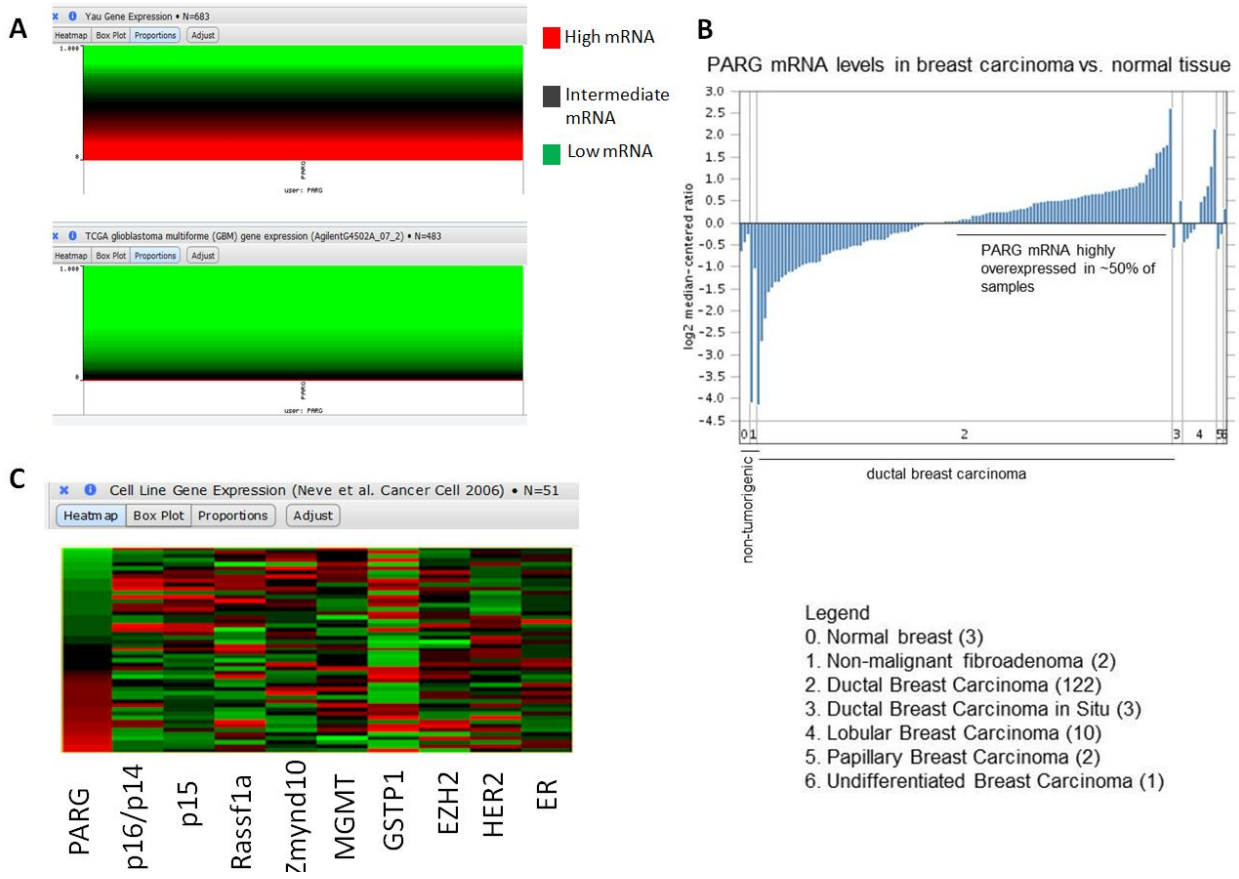

Figure 1. *PARG* mRNA is overexpressed in an array of breast cell lines and tumor tissues. **A.** The UCSC Cancer Browser reveals that approximately one third of breast cancer cell lines are positive for *PARG* mRNA (red) unlike other cancers such as glioblastoma multiforme where there is a generalized underexpression of *PARG* (green) in all existing cell lines. **B.** Data from the Oncomine database illustrates that approximately 50% of ductal breast carcinomas overexpress *PARG* mRNA in comparison to non-tumorigenic tissue. Other subtypes of breast cancer, likewise, have higher *PARG* mRNA in some cases. **C.** *PARG* mRNA levels were found to correlate well with underexpression of *p16/p14*, *p15*, *RASSF1a* and *ZMYND10* mRNA, but not with *MGMT*, *GSTP1*, *EZH2*, *HER2* and *ER* mRNA.

A

UCSC Cancer Browser Cell lines Encyclopedia					
High Parg mRNA			Low Parg mRNA		
Cell line	luminal	basal	Cell line	luminal	basal
CAL120	+		HCC1569		+
HCC1428	+		HCC1937		+
HCC1500	+		CAL851		+
CAL51			HS578T		+
HCC38		+	HCC70		+
BT483	+		EVSAT		
BT474	+		AU565	+	
T47D	+		BT20		+
JIMT1		+	MDA-MB-157		+
HCC202	+		HS739T		
MDA-MB-134VI	+		HS742T		
HCC1143		+	HS281T		
KPL1	+		MDA-MB-453	+	
CAL148	+		HS606T		
HCC1187		+	HCC1395		+
SKBR3	+		YMB1		
MDA-MB-436		+	MDA-MB-175VII	+	
MDA-MB-361	+		HCC2157		+
HS274T			MDA-MB-415	+	
EFM19	+		HCC2218	+	
			BT549		+
			UACC893	+	
			HS343T		
			DU4475		
			HCC1806		
			MDA-MB-231		+
			HCC1954		+
Total	13	5	Total	6	12

B

UCSC Cancer Browser Neve					
High Parg mRNA			Low Parg mRNA		
Cell line	luminal	basal	Cell line	luminal	basal
SUM 185PE	+		HCC1937		+
ZR75B	+		Sum149PT		+
MDA-MB-361	+		ZR7530	+	
MDA-MB-231		+	600MPE	+	
UACC812	+		SUM225cwn		+
HCC1500	+		sum1315mo2		+
HCC1007	+		CAMA-1	+	
HCC38		+	HCC202	+	
T47D	+		HCC1569		+
BT474	+		MDA-MB-157		+
BT483	+		HCC3153		+
MDA-MB-175-VII	+		LY2	+	
HCC1428	+		HCC2157		+
Sum190PT	+		HS578T		+
HCC2185	+		MDA-MB-435		+
			HCC1954		+
			HCC1143		+
			SUM159PT		+
			SUM44PE		
			HCC70		+
			HBL100		+
			SKBR3	+	
			MDA-MB-453	+	
Total	13	2	Total	7	15

Figure 2. Classification of *PARG* high and low breast cancer cell lines into luminal or basal subtypes. Cell lines were stratified according two categories based on high (red) or low (green) *PARG* mRNA expression based on the information provided from **A.** the Cell line encyclopedia and **B.** the Neve expression file from the UCSC cancer browser. There were further characterized as being basal or luminal based on descriptions from the literature.

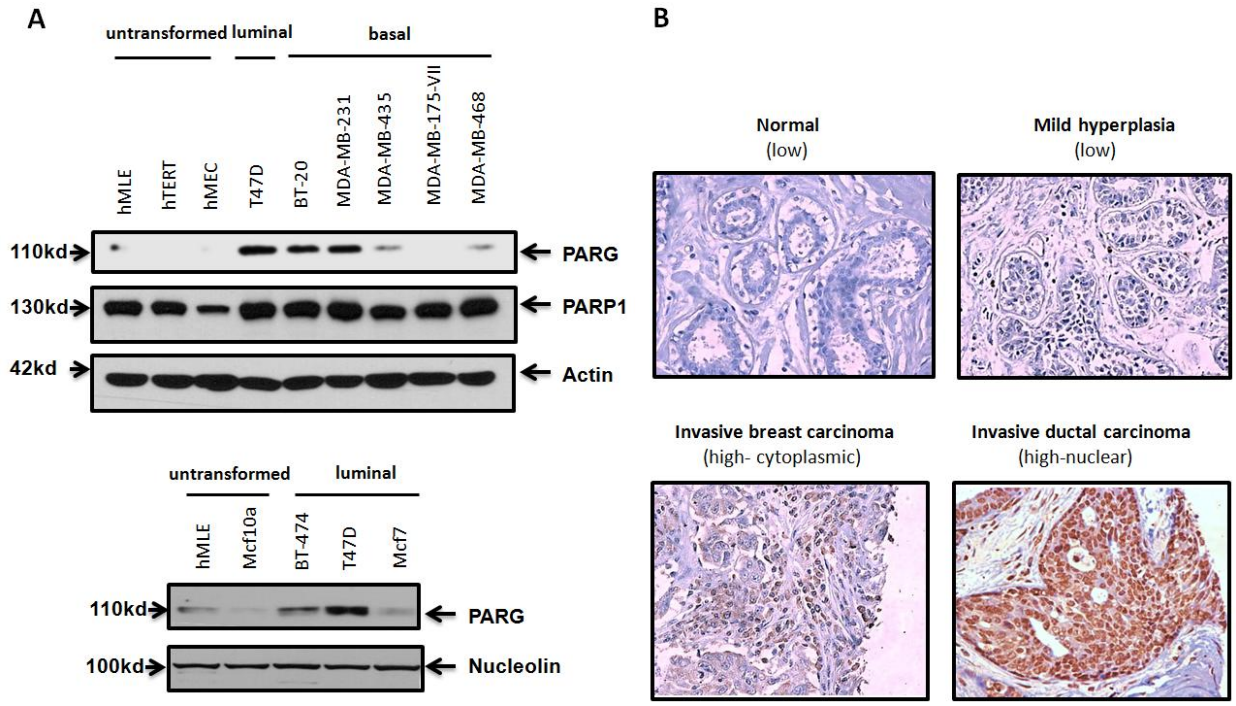

Figure 3. Parg protein is overexpressed in cell lines and in clinical samples. **A.** Higher levels of Parg protein are detected in both luminal and basal breast cancer cell lines in comparison to untransformed control cells. **B.** High Parg levels are commonly observed in invasive breast carcinoma samples, with low expression generally found in normal tissue.

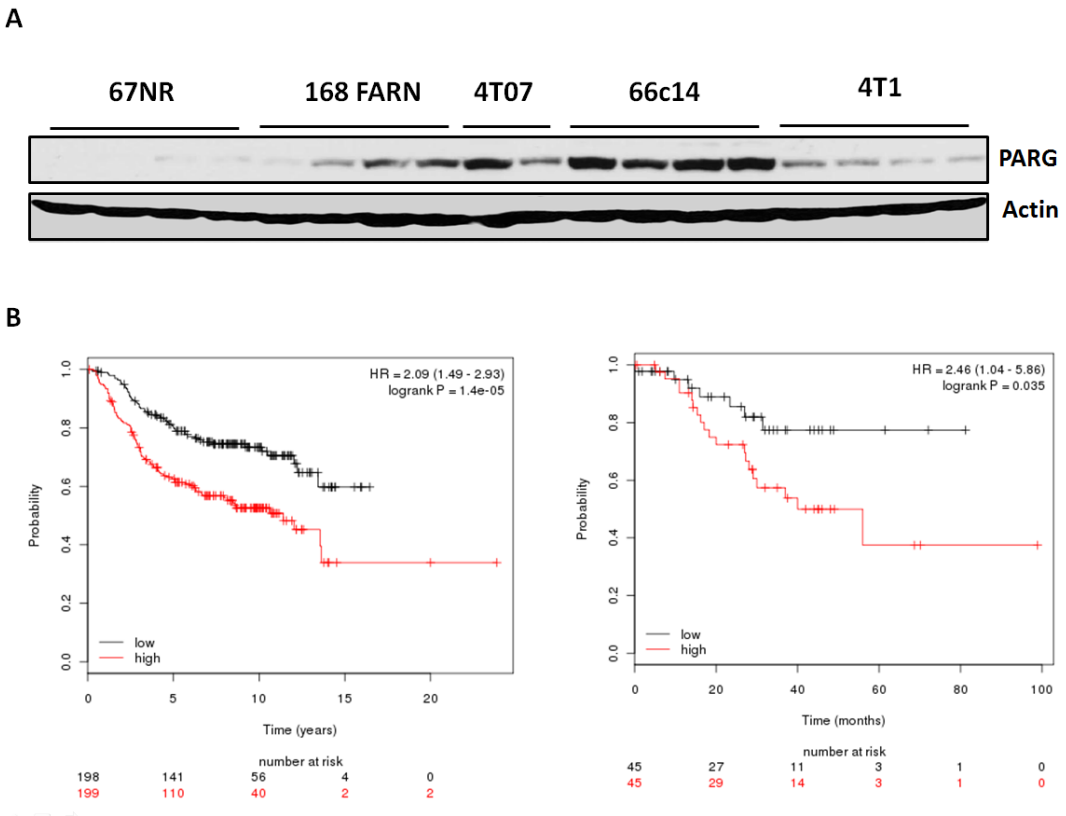

Figure 4. Parg is overexpressed in more aggressive stages of a mouse breast tumor model and correlates with poor clinical outcomes. A. Parg protein expression correlates well with advanced stages of breast cancer. 67NR cells are spontaneously arising, primary tumor cells; 168 FARN cells have acquired the ability to disseminate to the lymph; 4T07 are capable of dissemination to lymph and a small number can disseminate to the lungs, but they are incapable of clonogenic growth; 66c14 are highly metastatic to lung; 4T1 are highly metastatic to lung and liver. **B.** Kaplan-Meier curves displaying relapse-free survival based on Parg expression. Survival curves of node negative luminal B patients with high (red) or low (black) Parg levels (left) and of stage 1 and 2 ovarian cancer patients (right).

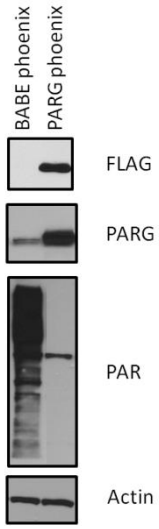

Figure 5. The effect of a transient Parg transfection on cellular PARylation. A transient transfection of Hek293t phoenix cells drastically reduces levels PARylation in the cell.

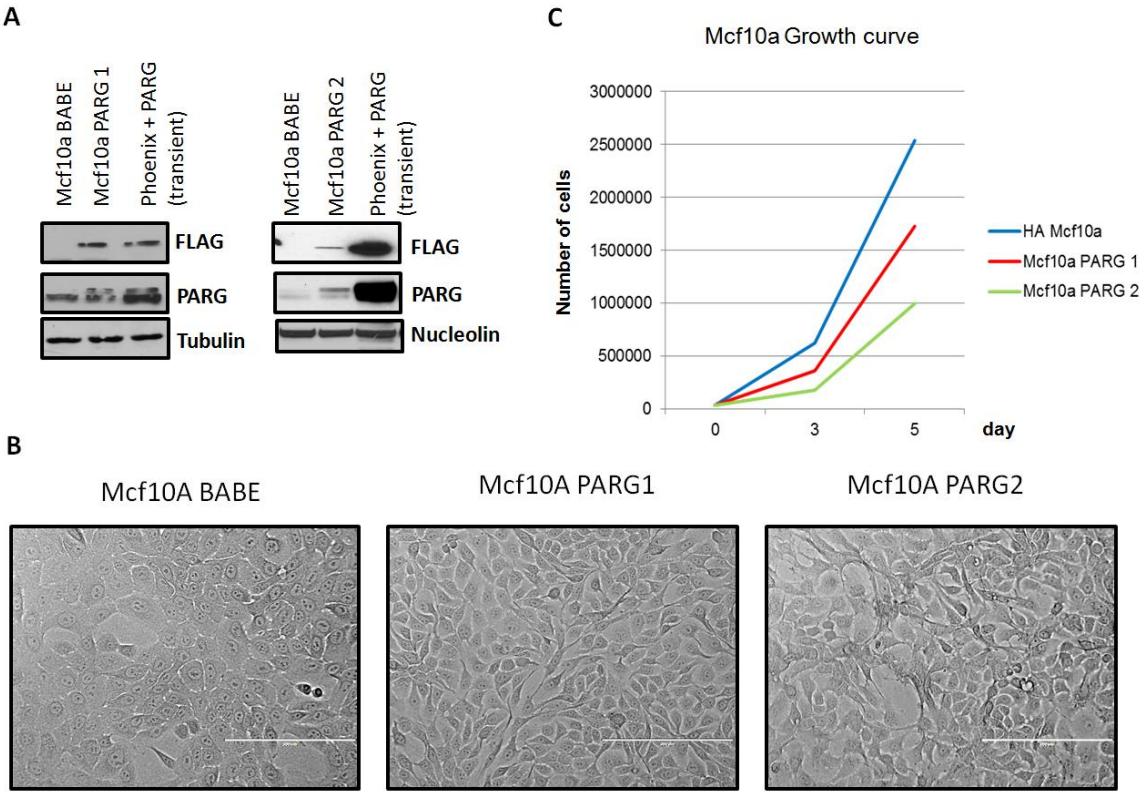

Figure 6. Parg overexpressing Mcf10a cells exhibit changes in morphology and growth rate. A. Mcf10a stable cell lines overexpress Flag-tagged Parg protein. **B.** Parg overexpression promotes a fibroblast-like morphology in Mcf10a cells. **C.** Parg overexpression reduces the growth rate of cells.

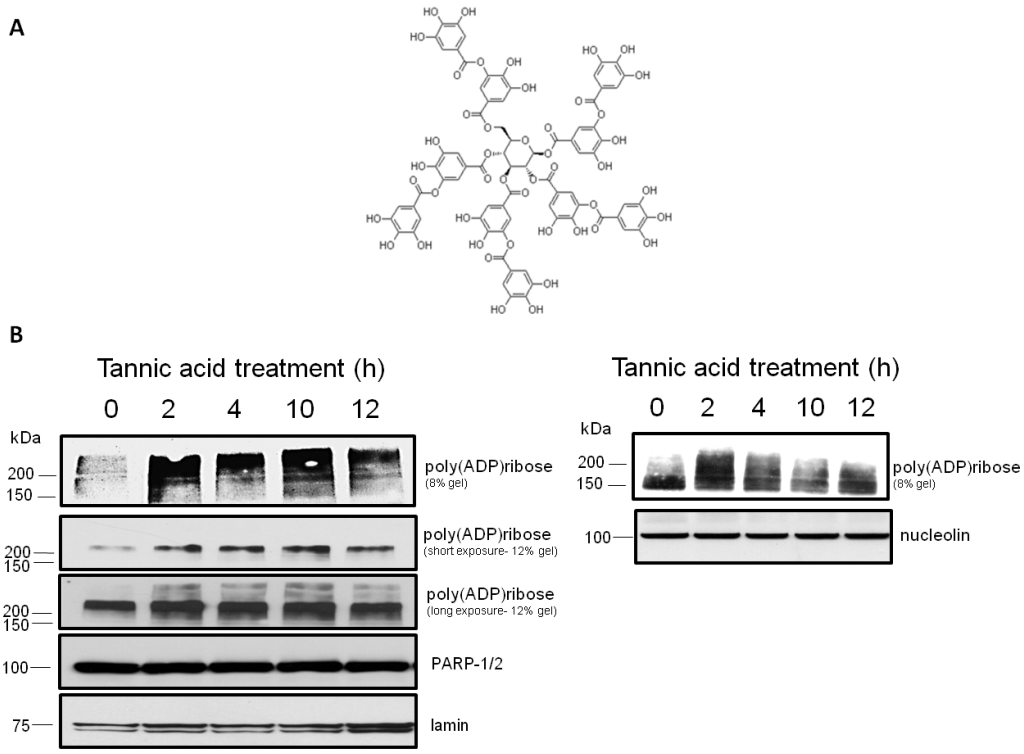

Figure 7. Tannic acid affects cellular PARylation. A. The structure of tannic acid. **B.** Western blot analysis revealing an increase in protein PARylation in T47D (left) and MDA-MB-468 cells (right) in response to 10 uM tannic acid.

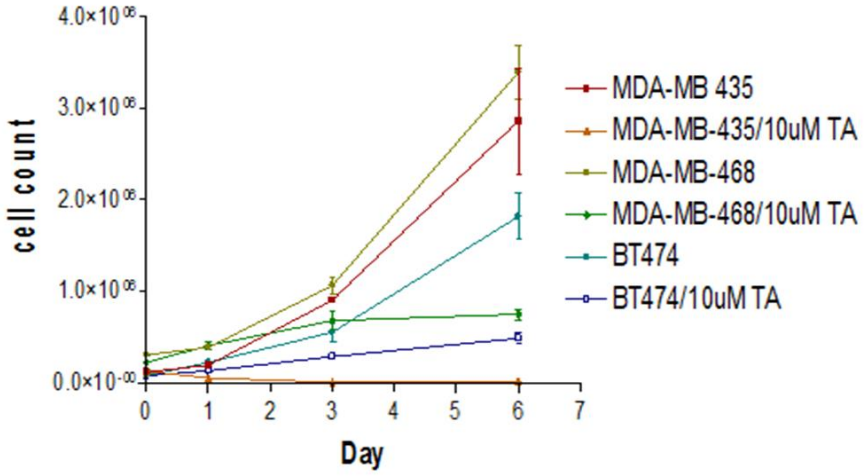

Figure 8. The growth of triple-negative and luminal breast cancer cell lines is inhibited by tannic acid. The Parg inhibitor tannic acid (10 uM) arrests the growth of breast cancer cells, including the triple-negative breast cancer cell lines MDA-MB-435 and MDA-MB-468, with a more subtle effect on the luminal BT-474 cells.

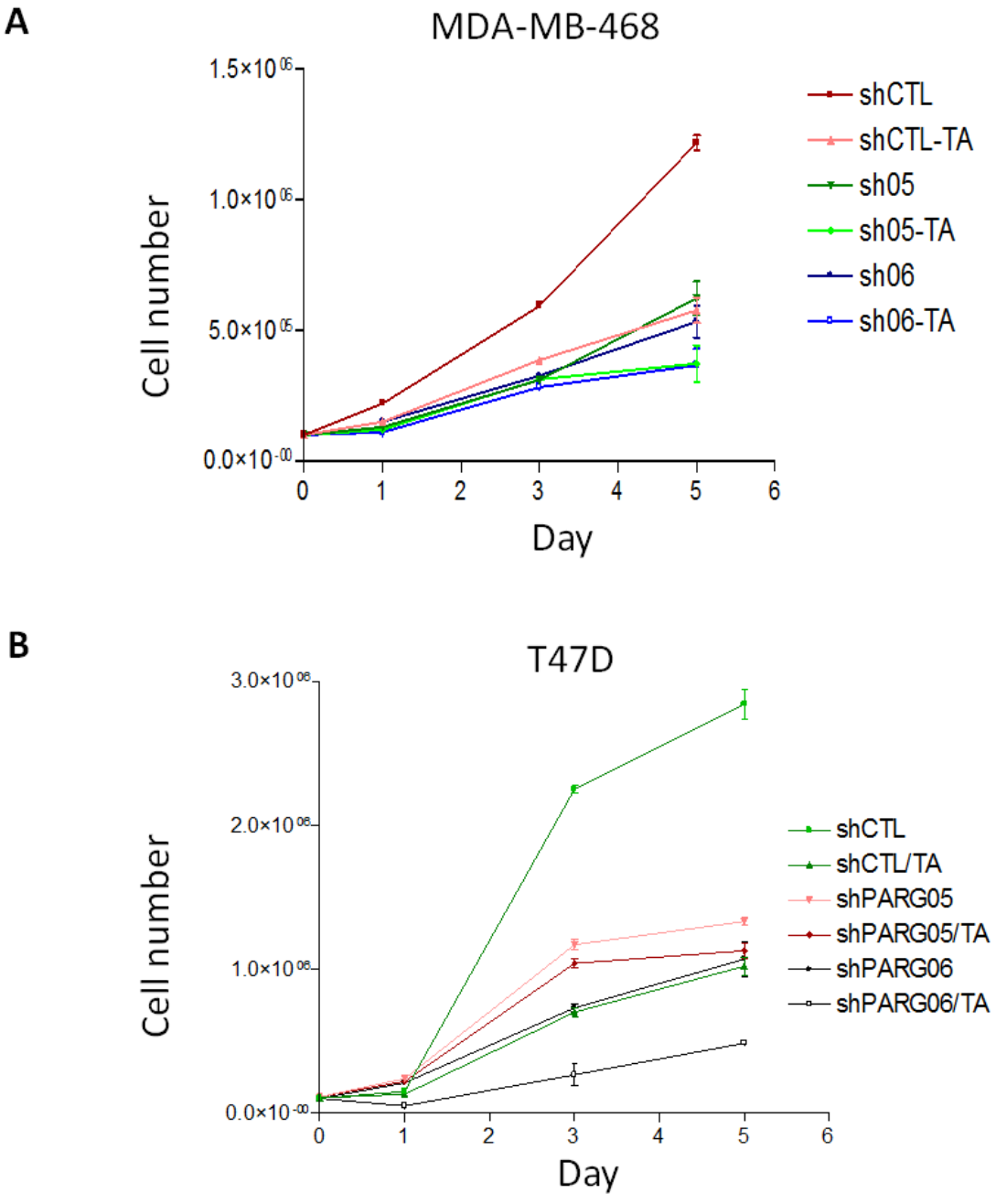

Figure 9. Tannic acid mediates cell growth inhibition through PARG. Tannic acid (10uM) treatment slows the growth of **A.** MDA-MB-468 and **B.** T47D cells. Knockdowns of Parg exhibit a similar decrease in cell growth as tannic acid treated cells. Moreover, treating Parg knockdown cells with tannic acid does not provide any further impact on slowing cell growth**.**

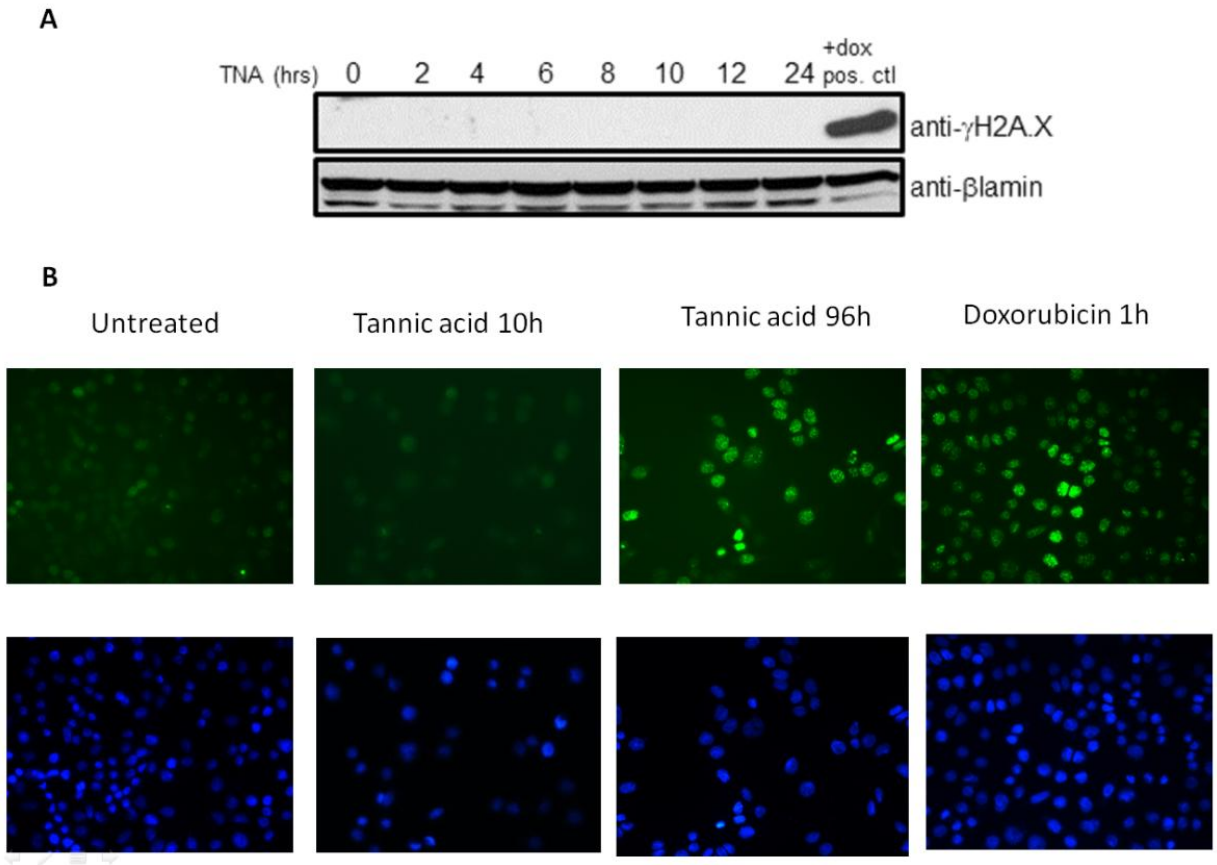

Figure 10. Tannic acid does not induce DNA damage over short time points. A. Western blot analysis of T47D cells reveals that DNA damage is not present following short exposures to 10uM tannic acid. **B.** Immunofluorescence staining of MDA-MB-468 cells treated with tannic acid. Green staining is representative of phospho-H2AX (Ser 139); Nuclei were visualized with 4′,6-diamidino-2-phenylindole staining (blue).

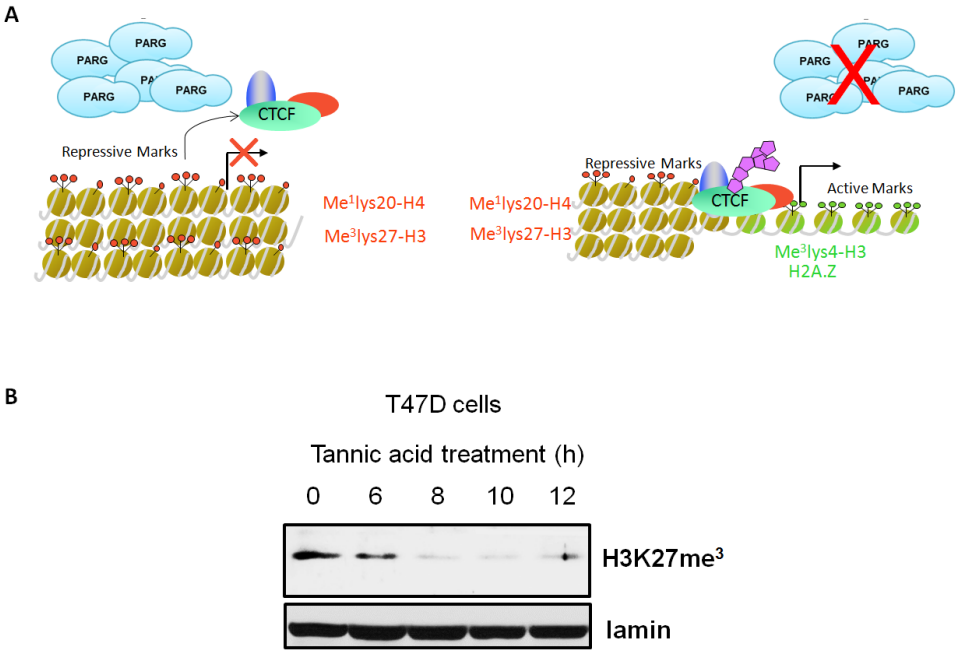

Figure 11. Tannic acid reduces the levels of the repressive H3K27me3 histone modification in T47D cells. A. Our model proposes that Parg inhibition would restore a chromatin boundary, reinstating activating histone marks at gene loci throughout the genome at the expense of repressive histone marks. **B.** Tannic acid treatment decreases the repressive histone mark H3K27me3 in T47D cells.

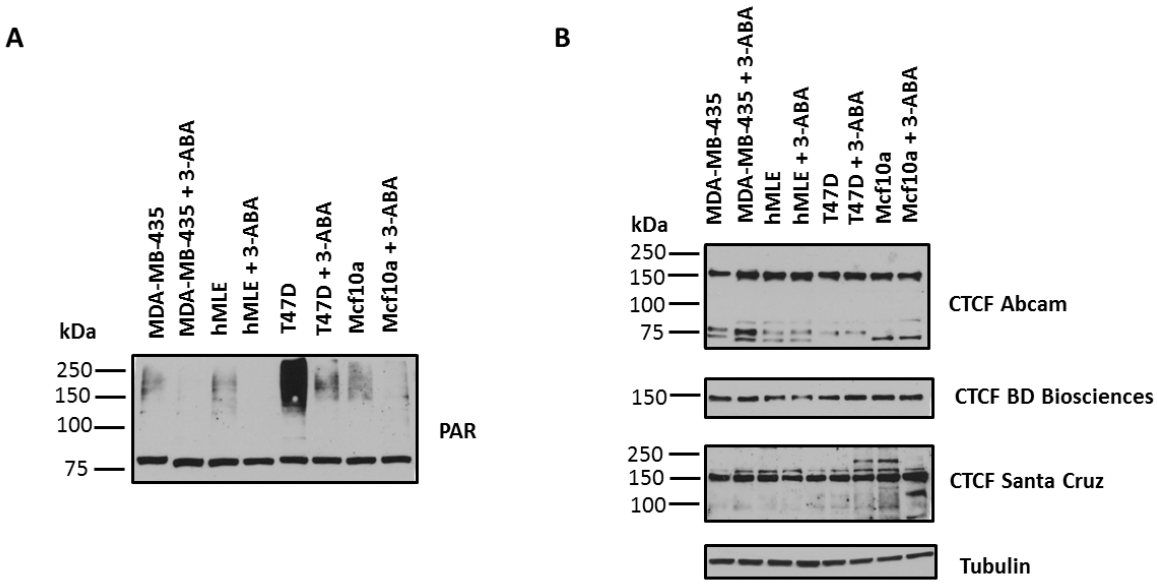

Figure 12. PARylated Ctcf cannot be directly tested by Western blot. A. 5mM 3-aminobenzamide (3-ABA) treatment reduces levels of cellular PARylation. **B.** Probing different cell lines with commercially available Ctcf antibodies.

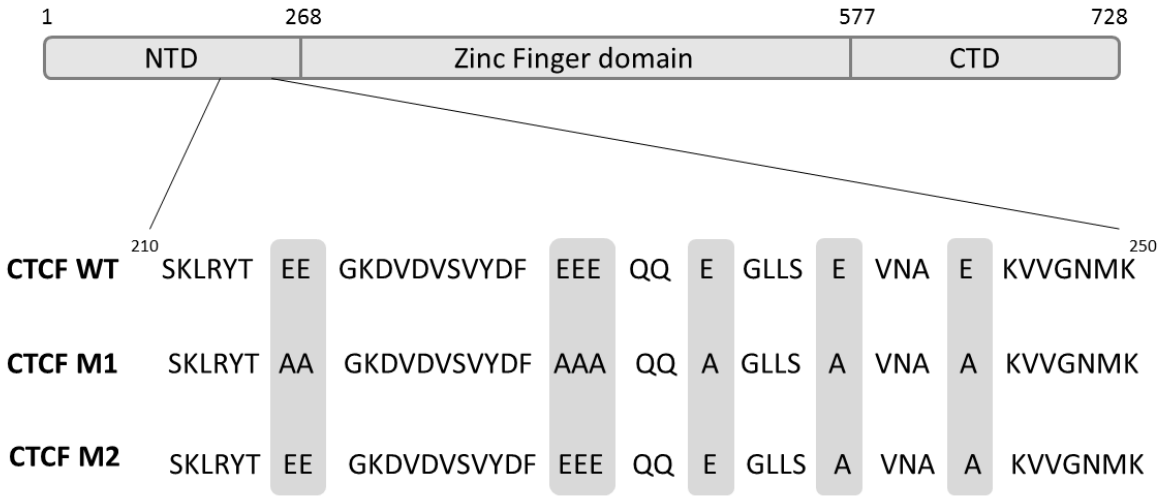

Figure 13. Ctcf mutant constructs. Ctcf mutants were targeted for amino acid substitution in the N-terminal domain of the protein. The amino acid sequence in this domain is presented with the altered residues highlighted in grey.

Figure 14. Ctcf mutant 2 is deficient in PARylation. Hek293t cells were transiently transfected with Ctcf wild-type and mutant plasmids 1 and 2, as well as the empty vector control. Cell lysates were then immunoprecipitated for the PAR modification and probed for HA-tagged Ctcf.

8. DISCUSSION

8.1| Parg is overexpressed in breast cancer

Among the important findings in this study is a significant correlation between Parg overexpression and breast cancer. Whether considering mRNA or protein levels, there is a striking overabundance of Parg in breast cancer cell lines and tumor tissue, as well as in more aggressive stages of a mouse tumor model. These results are a compilation of our own data with online bioinformatic databases. Our work clearly illustrates that non-transformed cell lines have lower Parg expression than transformed cell lines. In agreement with the bioinformatics data showing enrichment of Parg mRNA in luminal cell lines, an enrichment of Parg protein was also detected in cells of this subtype. Interestingly, while basal cell lines were found to be deficient in Parg mRNA according to the bioinformatics data, several of the basal cell lines tested by Western blot had significantly high levels of Parg protein. This suggests that the bioinformatics data concerning Parg mRNA may be an underestimate of the actual proportion of cancers with high Parg levels. Thus, Parg may have broader impact than the 30-50% of breast cancers that were found to overexpress Parg at the mRNA level.

In accordance with the Western blot analysis of cell lines, our immunohistochemistry data clearly revealed a significant proportion of clinical tumors samples with high Parg expression. Interestingly, several of the "normal" breast tissue provided also tested positive for Parg protein. We believe that this may be due to the fact that the 'normal' samples provided in the purchased arrays was tissue taken from just outside the tumor site. Our group questions whether these samples can be considered normal, given that it has been shown that breast cancer recurrence often occurs within a margin near the original tumor site (Li, Moore et al. 2002). This peripheral area already has a genetic and epigenetic predisposition for mutations and cancer. Consequently, we have postponed further analysis of this data until we have obtained a sufficient amount of mammary reduction tissue for comparison. Nevertheless, this data supports the fact that clinical breast tumors possess high Parg protein which may play a role in the malignancy of the disease. Moreover, this analysis has unveiled differences in localization of Parg protein, having been detected in both nuclear and cytoplasmic compartments which are likely to impact

the biology of cells in different ways. These differences may be an important consideration when testing the efficacy of drugs on different cell lines, for example (described later). Not only have we detected a significant proportion of breast tumors with an enrichment of Parg, but, using the Kaplan-Meier plotter resource online, we were able to associate elevated Parg expression to worse clinical outcomes in luminal B, node negative breast cancer. Similar findings were detected in ovarian cancer, suggesting that the effects of Parg may extend beyond breast cancer alone. Considered together, the data presented here provided a strong incentive to pursue Parg as a potential driver of breast cancer and target for therapy.

The main rationale for studying Parg in breast cancer was that this protein may remove the PARylation from the boundary forming Ctcf protein, contributing to the silencing of TSGs and ultimately, cancer progression. As such, it was important to assess the correlation of Parg mRNA with the expression of other genes that have been tested by microarray. Using the UCSC Cancer Browser as a tool, we discovered that the expression of TSGs like $p16^{INK4a}$, $p15^{INK4b}$ and *RASSF1a* inversely correlates with Parg levels. This fits well with our model that proposes that Parg mediates the silencing of TSGs. *RASSF1a* and $p16^{INK4a}$ silencing described in the literature was found to correlate well with Parg detection by Western blot. Cell lines including T47D and MDA-MB-231 have high Parg protein with epigenetically silenced or deleted $p16^{INK4a}$ and *RASSF1a*. Cell lines with intermediate levels of Parg expression, including MDA-MB-435 and MDA-MB-468 cells, however, were found to have unmethylated promoters at these loci (Bisogna, Calvano et al. 2001). Thus, there may be a link between the levels of Parg in cells and their expression of some key TSGs as those described above. Among the other factors considered was the *ZMYND10* gene, juxtaposed to Rassf1a in the genome. Interestingly, there is a Ctcf target site between these genes that demarcates a pronounced chromatin boundary between these factors. This boundary, however, does not extend to the promoter region of *ZMYND10*. Hence, Ctcf binding is not correlated to *ZMYND10* expression (Chang, Hsu et al. 2010). According to the data obtained from the Cancer Browser, however, there seems to be a strong positive correlation between *RASSF1a* and *ZMYND10*. This suggests that perhaps there is some coordination between these genes, despite previous reports. Interestingly, there was no obvious correlation between the expression of *ER* and *HER2* with Parg, suggesting that Parg overexpression does not necessarily equate to a triple-negative status in the cell. Thus, if indeed

Parg is characterized as a driver of cancer, it may possibly serve as a target in some triple-negative breast cancer cases.

8.2| Biochemical Parg overexpression

Despite the intriguing correlative evidence between Parg overexpression and TSG silencing, it still remains necessary to establish a causal link between the two. While we were successful in inducing high Parg levels in Mcf10a cells, the Parg transgene was silenced after only a few short passages in culture. This suggests that this factor may have deleterious effects in the cell and that there is a strong selective pressure to reduce the expression of the gene. Interestingly, we were able to detect a change in morphology in the Parg-overexpressing cells that indicated an EMT change. This was matched with a decrease in cell growth. This has yet to be reproduced and it may simply be a cell type-specific event. Future studies should be focused on generating a larger variety of stable Parg overexpressing cell lines that were originally untransformed (including Mcf10a, hMLE and HMEC cells). In turn, these should be tested for a variety of growth and transformation assays including growth curves, soft agar, matrigel invasion and focus forming assays. We speculate that Parg overexpression may suffice to transform cells. Alternatively, if no phenotype is observed, we hope to overexpress Parg in conjunction with another oncogene such as Her2. Cells require multiple hits in order to go from a benign to a malignant phenotype. The Mcf10a cells we hope to use for these experiments have already been in culture for quite some time, and have therefore already acquired some mutagenic hits in order for them to be maintained in cell culture. These changes may be sufficient to induce transformation when combined with Parg overexpression. However, the overexpression of Parg and Her2, we speculate, may synergize to yield a more pronounced biological effect and induce malignancy and transformation in the Mcf10a cells. It would also be interesting to study the effect of Parg overexpression in conjunction with other oncogenes in various cell lines for comparison.

For these overexpression studies, it may also be interesting to set up an inducible overexpression system where the levels of Parg can be regulated by the addition of a drug. This may help avoid problems with spurious silencing of the transgene, as was the case in the experiments conducted to date. This system will also provide a better negative control for normal

Parg levels as the same cells will be used for the Parg overexpression experiments except that these would not be subject to drug treatment. This will eliminate any differences that may occur from differential integration of the transgene in the genome between the empty vector and the one containing the Parg gene. At the same time, cell lines overexpressing the mutant forms of Ctcf that are incapable of being modified by PARylation should be established in similar cell lines and tested for similar transformation properties.

If a phenotype is observed in these cells, we hope to characterize the effects on gene transcription with microarray experiments. We expect that Parg overexpressing cell lines may have a negative effect on gene transcription. In particular, it would be of interest to monitor the changes at TSG loci including $p16^{INK4a}$ and *RASSF1a*, known to be flanked by Ctcf binding sites. Comparing the effect at these loci with that occurring at oncogene loci may reveal some specificity to the effect of Parg on transcription. Specifically, we hope to test for changes in epigenetic marks at these genes and others, through ChIP-seq experiments.

8.3| Inhibition of Parg through tannic acid slows the growth of breast cancer cells

The ultimate goal of this study is to determine whether chemically inhibiting Parg may provide some relief to women with breast cancer. To date, tannic acid is currently the best available Parg inhibitor on the market. Although tannic acid has some off-target effects such as the epidermal growth factor receptor (Egfr) (Yang, Wei et al. 2006), unpublished results from our lab suggest that Egfr is not the main target of tannic acid in breast cancer cells. The lack of a better drug is likely due to the absence of a crystal structure of the protein. However, a recent publication in Nature (Slade, Dunstan et al. 2011) has revealed the structure of the protein which will likely spur the development of new Parg inhibitors in the near future. In the meantime, for the purpose of this study, we chose to work with the non-specific Parg inhibitor, tannic acid, in hopes of characterizing the effect of Parg inhibition on cell growth. This drug was particularly attractive given its non-toxic profile (Fujiki, Yoshizawa et al. 1992).

Before testing the effect of tannic acid on cell growth, it was first necessary to test the ability of this drug to inhibit the protein of interest. In order to ensure that the tannic acid was acting through Parg, we assessed changes in global PARylation levels in response to the drug.

Western blot analysis of PARylation levels in the cell in response to tannic acid revealed an increase in PARylation over a short time period for MDA-MB-468 and T47D cells, consistent with an effect of Parg inhibition. This data was supplemented with immunofluorescence data showing that DNA damage caused by tannic acid is insignificant over these time points. This served as an important control to ensure that the increase in PARylation observed with tannic acid was indeed an effect of Parg inhibition and not due to overactivity of Parp in response to DNA damage. At longer time points, however, phospho-H2A.X foci begin to appear. This may have to do with a role for Parg in the DNA damage repair response. DNA damage is sufficient to slow the rate of cellular proliferation, to allow sufficient repair of damage before proceeding with the cell cycle (Noguchi 2010). In addition to an impact on DNA damage, we also were able to characterize an epigenetic effect for Parg. In particular, we observed that Parg inhibition reduces the expression of the repressive histone mark H3K27me3. We speculate that this effect is due to the reestablishment of Ctcf chromatin boundaries (maintained by PARylated Ctcf). This, in turn, prevents the spread of heterochromatin and ultimately reduces repressive histone marks. However, the global deficiency of H3K27me3 could also result from an inhibitory effect of tannic acid on EZH2, the protein responsible for adding the H3K27me3 mark. Although there have not been any reports showing an effect of tannic acid on the polycomb repressor protein, we cannot exclude this possibility from the results presented here. Together, our results suggest that there is likely a role for both DNA damage and epigenetic reactivation of TSGs in slowing cell growth of cells in response to tannic acid, with the former taking precedence at short time points and the latter following longer exposures to the drug.

The mechanistic description provided above is matched by findings that tannic acid effectively slows the growth of breast cancer cell lines in culture. Interestingly, while the triple-negative MDA-MB-468 and MDA-MB-435 cells have intermediate expression levels Parg protein, these cells are very sensitive to tannic acid. The luminal BT-474 cells, while also having intermediate levels of Parg protein, are less sensitive to the drug. One important consideration is that we probed for total cellular Parg protein, and did not distinguish between nuclear and cytoplasmic forms of Parg. In the literature, the 111kDa Parg isoform has been found to be mainly nuclear while the 99kDa and 102kDa isoforms were mainly cytoplasmic (Meyer-Ficca, Meyer et al. 2004). By Western blot, it is difficult to distinguish between the three forms and depending on the cell line, these isoforms may not be completely restricted to the cellular

compartments described for them. Our immunohistochemistry analysis alluded to the possibility that the Parg protein from the whole cell lysates detected by Western blot may not be strictly nuclear. Thus, perhaps separating nuclear and cytoplasmic fractions before probing by Western blot and stratifying Parg based on cellular localization may provide a better predictive tool as to which cells will be more sensitive to tannic acid and Parg inhibitors. Alternatively, it is possible that there is an underlying genetic background to the triple-negative cells that renders them more sensitive to Parg inhibition than the luminal cells. This notion, however, should be tested by screening a wider variety of basal, triple-negative or luminal cell lines for more generalizable results. Perhaps, in this way, a more precise pattern of tannic acid sensitivity will arise which would be useful for clinical purposes.

The effect of tannic acid on cell growth was mimicked by Parg knockdowns in the triple-negative MDA-MB-468 and the luminal T47D cells. Indeed, depletion of Parg reduces the rate of cellular proliferation to one that is comparable to that obtained with tannic acid treated cells. Treatment of the knockdowns with tannic acid does not further slow the rate of cellular proliferation, suggesting that tannic acid is working through Parg to mediate cell growth arrest. Again, it would be interesting to screen a wider variety of cell lines having varying degrees of Parg expression to see whether a correlation can be established between levels of Parg and their sensitivity to Parg depletion in slowing cell growth.

8.4| The detection of PARylated Ctcf

We speculate that one of the main effects of Parg overexpression in the cell may be to disrupt the PARylation of Ctcf. For this purpose, it was important to establish an efficient method of detecting this post-translational modification of such a protein. This has led us to attempt to reproduce findings by Klenova et al. that reported two detectable forms of Ctcf by Western blot, one at a molecular weight of 130kDa and the other at 180kDa (Docquier, Kita et al. 2009). Specifically, the higher molecular weight form was described as being the PARylated form. However, direct evidence of this claim was never presented by the group, necessitating further investigation in our lab. Our data, screening several cell lines in the presence or absence of 3-ABA treatment did not show any evidence of this 180kDa band claimed by Klenova's group. This has led us to question the validity of their results. Using this rationale, they go on to

claim that the non-PARylated form of Ctcf (130kDa) is only present in ductal breast tumors, although not allowing for the possibility of a differentially spliced form of the tumor. For more conclusive results, it may be necessary to generate Ctcf knockdowns to see if there truly is disappearance of the band at 180 and 130kDa, although already doubts are raised concerning the results presented by the group. One interesting possibility we conceived is that during the lysis of cells, Parg may artificially degrade the PAR modifications in the lysate, leaving a seemingly shorter ADP ribose chains than is actually present in the cell. As such, the single 150kDa protein we detect may be Ctcf with only a short PAR chain whereas the reality may be a protein with a longer modification that migrates at a size of 180 kDa. To avoid this possible artifact, it may be necessary to lyse cells in the presence of PARG inhibitors, as is done with phosphatase and protease inhibitors. Perhaps, with the necessary inhibitors added to the lysis buffer, it may be possible to retain long PAR chains attached to Ctcf which could in turn, be directly detected by Western blot. Indeed, co-immunoprecipitation experiments should also likely be carried out in the presence of Parg inhibitors given that undesired dePARylation within the cell lysate could remove much of the polyADP ribose modification, preventing a higher molecular weight form from being detected and perhaps disrupting some of the molecular interactions that exist in the cell.

8.5| Ctcf mutants deficient in PARylation

The ability to detect PARylation of Ctcf was important to characterize the ability of the Ctcf mutants generated to be modified by this post-translational change. Using immunoprecipitation, the results suggested that Ctcf mutant 2 is PARylation deficient. Interestingly, however, this mutant possesses fewer changes than mutant 1. In fact, Ctcf mutant 1 possesses the same core as mutant 2 as well as additional modifications. Nevertheless, mutant 1 remains PARylated to a similar extent as the wild-type protein. This is counterintuitive, suggesting that the addition of poly(ADP)ribose chains is not as straightforward as phosphorylation or acetylation that targets one specific residue. Future experiments should be focused on screening different cell lines to see if there are cell-specific differences in the PARylation of the Ctcf mutants.

9. CONCLUSION

In conclusion, our analyses have established a relation between Parg overexpression and breast cancer, uncovering hints that Parg may be involved in cell transformation. We have also found evidence that Parg inhibition can provide relief to breast cancer patients, particularly those with a triple-negative subtype. Thus, we propose that Parg is a new therapeutic target in breast cancer.

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